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(54) Title: METHOD FOR SOLUTION PHASE SYNTHESIS OF OLIGONUCLEOTIDES

## (57) Abstract

This invention discloses an improved method for the sequential solution phase synthesis of oligonucleotides. The method lends itself to automation and is ideally suited for large scale manufacture oligonucleotides with high efficiency.

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## METHOD FOR SOLUTION PHASE SYNTHESIS OF OLIGONUCLEOTIDES

### FIELD OF THE INVENTION

This invention relates to the field of nucleic acid chemistry. Specifically, this invention describes a novel method for preparing oligonucleotides. The method utilized herein for preparing said oligonucleotides is called PASS, an acronym for 5 Product Anchored Sequential Synthesis.

### BACKGROUND OF THE INVENTION

Until quite recently, oligonucleotides were not considered in any capacity other than strictly informational. Despite the fact that certain oligonucleotides were known 10 to have interesting structural possibilities (e.g., t-RNAs) and other oligonucleotides were bound specifically by polypeptides in nature, very little attention had been focused on the non-informational capacities of oligonucleotides. For this reason, among others, little consideration had been given to using oligonucleotides as pharmaceutical compounds.

There are currently at least three areas of exploration that have led to 15 extensive studies regarding the use of oligonucleotides as pharmaceutical compounds. In the most advanced field, antisense oligonucleotides are used to bind to certain coding regions in an organism to prevent the expression of proteins or to block various cell functions. Additionally, the discovery of RNA species with catalytic functions -- ribozymes -- has led to the study of RNA species that serve to perform intracellular reactions that will 20 achieve desired effects. And lastly, the discovery of the SELEX process (Systematic Evolution of Ligands by Exponential Enrichment) (Tuerk and Gold (1990) Science 249:505) has shown that oligonucleotides can be identified that will bind to almost any biologically interesting target.

SELEX is a method for identifying and producing nucleic acid ligands, 25 termed "nucleic acid antibodies," e.g., nucleic acids that interact with target molecules (Tuerk and Gold (1990) Science 249:505). The method involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection theme, to achieve virtually any desired criterion of binding affinity and selectivity.

30 The use of antisense oligonucleotides as a means for controlling gene expression and the potential for using oligonucleotides as possible pharmaceutical agents

has prompted investigations into the introduction of a number of chemical modifications into oligonucleotides to increase their therapeutic activity and stability. Such modifications are designed to increase cell penetration of the oligonucleotides, to stabilize them from nucleases and other enzymes that degrade or interfere with the structure or activity of the 5 oligonucleotide analogs in the body, to enhance their binding to targeted RNA, to provide a mode of disruption (terminating event) once sequence-specifically bound to targeted RNA and to improve their pharmacokinetic properties.

Recent research has shown that RNA secondary and tertiary structures can have important biological functions (Tinoco *et al.* (1987) *Cold Spring Harb. Symp. Quant. Biol.* 52:135; Larson *et al.* (1987) *Mol. Cell. Biochem.* 74:5; Tuerk *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:1364; Resnekov *et al.* (1989) *J. Biol. Chem.* 264:9953). PCT Patent Application Publication WO 91/14436, entitled "Reagents and Methods for Modulating Gene Expression Through RNA Mimicry," describes oligonucleotides or oligonucleotide analogs which mimic a portion of RNA able to interact with one or more proteins. The 10 oligonucleotides contain modified internucleoside linkages rendering them nuclease-resistant, have enhanced ability to penetrate cells, and are capable of binding target 15 oligonucleotide sequences.

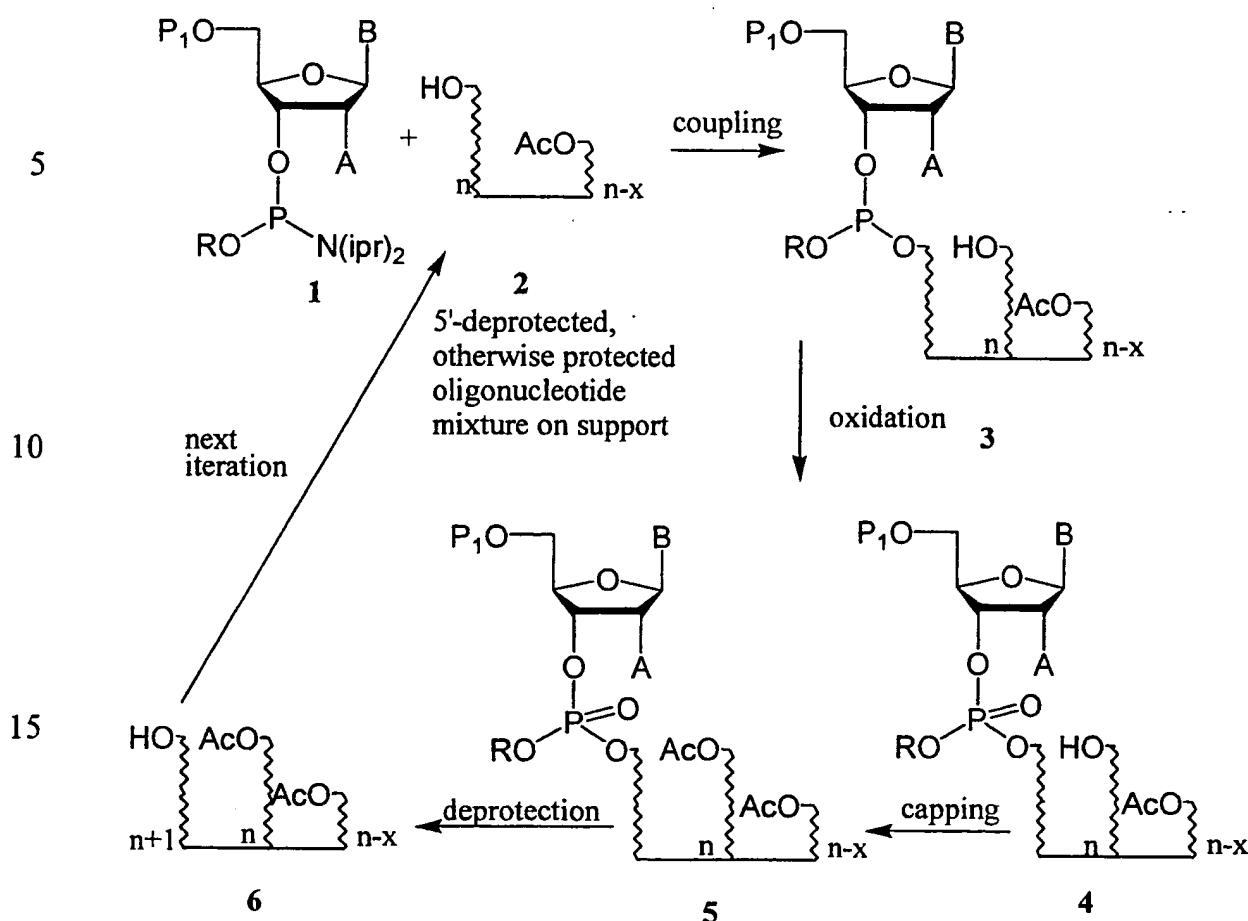
Although there has been a fair amount of activity in the development of modified oligonucleotides for use as pharmaceuticals, little attention has been paid to the 20 preparation and isolation of these compounds on a scale that allows clinical development. The conventional laboratory scale 1  $\mu$ mole automated oligonucleotide synthesis does not provide a sufficient amount of the compound of interest to enable clinical development. For clinical development oligonucleotides must be produced in gram-scale to multigram scale amounts at a minimum. Although there are reports of large-scale oligoribonucleotide 25 syntheses in the literature, the term "large-scale" has been applied to the 1 to 10  $\mu$ mole scale, rather than gram-scale or kilogram-scale amounts. (Iwai *et al.* (1990) *Tetrahedron* 46:6673-6688).

The current state of the art in oligonucleotide synthesis is automated solid phase synthesis of oligonucleotides by the phosphoramidite method, which is illustrated in 30 Scheme 1. (Beaucage and Iyer (1992) *Tetrahedron* 48:2223-2311; Zon and Geiser (1991) *Anti-Cancer Drug Design* 6:539-568; Matteucci and Caruthers (1981) *J. Am. Chem. Soc.*

103:3185-3191). Briefly, the 3'-terminal nucleoside of the oligonucleotide to be synthesized is attached to a solid support and the oligonucleotide is synthesized by addition of one nucleotide at a time while remaining attached to the support. As depicted in Scheme 1 a nucleoside monomer is protected (P<sub>1</sub>) and the phosphoramidite is prepared (1). The 5 phosphoramidite (referred to as the 5'-protected monomer unit) is then covalently attached to the growing oligonucleotide chain (2), via a phosphite triester linkage, through the 5'-hydroxy group of the ribose ring of the growing oligonucleotide chain to yield the oligonucleotide product (3), in which the majority of the growing oligonucleotide chain has been extended by one nucleotide, but a significant percent of chains are not extended. The 10 product (3) is then oxidized to yield the phosphate triester (4). Prior to the addition of the next base to the growing nucleotide chain, the 5'-hydroxyl group must be deprotected. As can be seen in Scheme 1 (compound 4), however, not all of the reactive sites on the solid support react with the 5'-protected monomer. These unreacted sites (referred to as failure sequences) must, therefore, be protected (referred to as capping) (5) prior to deprotection of 15 the 5'-hydroxyl group (6). Subsequent monomers, which have also been protected and converted to the phosphoramidite, are then sequentially added by coupling the 5'-end of the growing oligomer to the 3'-end of the monomer. Each coupling reaction extends the oligonucleotide by one monomer via a phosphite triester linkage. At each step -- and in the case of the initial reaction with the solid support -- there are reactive sites that fail to react 20 with the 5'-protected monomer, which results in oligonucleotides that have not been extended by one nucleotide monomer (failure sequences). When the synthesis is complete the desired oligonucleotide (6 (n+1 sequence)) is deprotected and cleaved from the resin, together with all of the failure sequences (n, n-x).

The yield of conventional solid phase oligonucleotide synthesis decreases 25 exponentially with the number of monomers coupled. This increases the difficulty of purifying the crude product away from the failure sequences. Additionally, even after high resolution purification has been achieved, it remains very difficult to verify the sequence and composition of the product, especially if it contains non-standard nucleotides.

## SCHEME 1



Automated oligonucleotide synthesis on solid supports is very efficient for the preparation of small amounts, 0.001 to 0.01 mmol, of a variety of sequences in a minimum amount of time with reasonable yield. It is, however, a highly inefficient process in terms of overall process yield based on input monomer. Typically a 16 fold excess of phosphoramidite is necessary per monomer addition. It has been recognized that the automated solid phase synthesis approach does not readily lend itself to be scaled to a level that allows efficient manufacture of oligonucleotide pharmaceuticals. (Zon and Geiser (1991) *Anti-Cancer Drug Design* 6:539-568).

The inefficiency of the solid phase synthesis is created to a large extent by the heterophase monomer coupling reaction and by the covalent attachment of both unreacted failure sequences and reaction product to the same support bead. In each cycle, 1-5% of the nucleotide bound to the support does not react with the activated monomer.

These unreacted compounds, referred to as failure sequences, as discussed above, must be blocked or capped in order to prevent the subsequent addition of monomers to incomplete oligonucleotides. The generation of failure sequences at every step of the synthesis produces a crude product contaminated with highly homologous byproducts, which must be 5 carried through to the final crude product (see Scheme 1, structure 6 (n, n-x)). As a result, purification of crude synthetic oligonucleotides to a state acceptable for clinical studies is extremely cumbersome and inefficient. To minimize the percent of failure sequences, a large excess of monomer (approximately 16 fold) is used.

A method to scale-up solid phase oligonucleotide synthesis using a higher 10 loaded polystyrene support was reported by Montserrat *et al.* (1994) *Tetrahedron* 50:2617-2622. This method, however, does not overcome the primary problem associated with solid phase synthesis, in that a considerable monomer excess is still required to minimize failure sequences. Additionally, the method does not provide consistently satisfactory yields.

In an attempt to decrease the excess of monomer needed to achieve coupling 15 and to achieve easy scaleability, Bonora *et al.* (1993) *Nucleic Acids Res.* 21:1213-1217, have investigated using polyethylene glycol (PEG) as a 3'-support that is soluble in the monomer coupling reaction. This method has been used to prepare oligonucleotides by phosphoramidite coupling, H-phosphonate condensation and phosphotriester condensation. (See Bonora (1987) *Gazzetta Chimica Italiana* 117:379; Bonora *et al.* (1990) *Nucleic Acids 20 Res.* 18:3155; Bonora *et al.* (1991) *Nucleosides & Nucleotides* 10:269; Colonna *et al.* (1991) *Tetrahedron Lett.* 32:3251-3254; Bonora and Scermin (1992) *Innovation Perspect. Solid Phase Synth. Collect. Pap., Int. Symp.*, 2nd, "Large Scale Synthesis of Oligonucleotides. The HELP Method: Results and Perspectives," pp. 355-358, published by Intercept, Andover, UK; Scermin and Bonora (1993) *Tetrahedron Lett.* 34:4663; Bonora 25 (1995) *Applied Biochemistry and Biotechnology* 54:3; Zaramella and Bonora (1995) *Nucleosides & Nucleotides* 14:809). The weakness of this approach is the unacceptably low recovery of support bound oligonucleotide after each reaction step. Additionally, this method does not address the problem of failure sequences that must be capped and carried through to the final product.

30 A polyethylene glycol-polystyrene copolymer support has also been used for the scale-up of oligonucleotide synthesis. (Wright *et al.* (1993) *Tetrahedron Lett.* 34:3373-

3376). At the 1 mmol scale a 96.6% coupling efficiency per monomer addition was reported for an 18mer DNA. Again, this method does not address the problem of failure sequences bound to the resin.

Zon *et al.* have suggested a block approach to the synthesis of 5 oligonucleotides, in which a library of dimer or multimer oligonucleotide fragments are prepared in solution and then coupled to each other. (Zon and Geiser (1991) *Anti-Cancer Drug Design* 6:539-568). The fragments are activated for coupling by differential 5'-deprotection and 3'-phosphorylation. Phosphotriester coupling has been suggested for fragment preparation. (Bonora *et al.* (1993) *Nucleic Acids Res.* 21:1213-1217). Due to the 10 comparatively low yield of phosphotriester coupling this approach has not been widely accepted.

In conventional oligonucleotide synthesis, the 5'-protecting group serves to prevent reaction of the 5'-hydroxyl group of one monomer with the phosphoramidite group of a second monomer during the coupling step. The 4,4'-dimethoxytrityl (DMT) group is 15 commonly used as the 5'-protecting group (Schaller *et al.* (1963) *J. Am. Chem. Soc.* 85:3821) of the 5'-protected monomer unit added during oligonucleotide synthesis. This group is chosen because of the ease and selectivity with which it can be removed from the 5'-oxygen of the oligonucleotide product prior to addition of the next 5'-protected monomer unit (for a review see, Beaucage and Iyer (1992) *Tetrahedron* 48:2223-2311). In solution, 20 deprotection of the 5'-DMT group is impaired by the reversibility of acid induced detritylation. In order to drive this reaction to completion, a scavenger of the free trityl cation is added for solution-phase detritylation (Ravikumar *et al.* (1995) *Tetrahedron Lett.* 36:6587). It has been recognized that the final 5'-terminal DMT group may serve as a hydrophobic handle which allows separation of the full-length product oligonucleotide from 25 shorter failure sequences by reverse phase chromatography. Additionally, highly hydrophobic analogs of the DMT group have been prepared to enhance the resolution of the separation of full length deprotected oligonucleotide product from failure sequences after complete solid phase synthesis (Seliger and Schmidt (1987) *Journal of Chromatography* 397:141). In another approach, a fluorescent trityl analog has been used for the 5'-terminal 30 protecting group during oligonucleotide synthesis to allow facile detection of full-length product in crude deprotected oligonucleotide (Fourrey *et al.* (1987) *Tetrahedron Lett.*

28:5157). Colored trityl groups were devised to allow monitoring of specific monomer additions during solid phase oligonucleotide synthesis (Fisher and Caruthers (1983) *Nucleic Acids Res.* 11:1589). Other modified trityl groups have been prepared for the purpose of changing or enhancing the selectivity with which the trityl group can be removed from the 5 oligonucleotide during solid phase oligonucleotide synthesis (for a review see, Beaucage and Iyer (1992) *Tetrahedron* 48:2223-2311).

To date, trityl groups which allow anchoring of the product to a resin or membrane during oligonucleotide synthesis in solution have not been designed. Additionally, trityl groups which can covalently react with a derivatized resin, membrane or 10 soluble polymer have not been reported.

The Diels-Alder reaction is a cycloaddition reaction between a conjugated diene and an unsaturated molecule to form a cyclic compound with the  $\pi$ -electrons being used to form the new  $\sigma$  bonds. The Diels-Alder reaction is an example of a [4 + 2] cycloaddition reaction, as it involves a system of 4- $\pi$  electrons (the diene) and a system of 15 2- $\pi$  electrons (the dienophile). The reaction can be made to occur very rapidly, under mild conditions, and for a wide variety of reactants. The Diels-Alder reaction is broad in scope and is well known to those knowledgeable in the art. A review of the Diels-Alder reaction can be found in Advanced Organic Chemistry (March, J., ed.) 761-798 (1977) McGraw-Hill, NY, which is incorporated herein by reference.

20 Cookson's diones -- 4 substituted 1,2,4 triazoline-3,5-diones (RTAD) -- (Cookson *et al.* (1967) *J. Chem. Soc.(C)* 1905; Cookson *et al.* (1971) *Org. Syn.* 51:121), are among the most reactive Diels-Alder dienophiles known. These dienophiles have been incorporated onto amino derivatized silica gel, by both covalent and ionic attachment, and used to purify mixtures containing diene impurities by Diels-Alder reaction of the diene 25 impurity with the dienophile derivatized solid support. (Keana *et al.* (1983) *Org. Chem.* 48:1982). For example, samples of cholesterol containing ergosterol, a 1,3 diene which has been shown to react in a Diels-Alder fashion with RTAD derivatives (Barton *et al.* (1970) *J. Chem. Soc. (D)* 939), were treated with an excess of 3-aminopropyl-silica gel derivatized with various sulfonated 4-aryl-1,2,4 triazoline-3,5-diones (TDA-silica gel) at 25°C. 30 Following reaction nearly quantitative yields of pure cholesterol were obtained. Other dienes

which were trapped by this TAD-silica gel include 8,10-dodecadienol, 1-chloro-, 2-chloro- and 9-bromoanthracene.

In another example, arylsulfonic acid derivatives of 4-aryl(2,6-diisopropyl)-1,2,4-triazoline-3,5-diones were prepared and incorporated onto an amino silica gel. This 5 TAD-silica gel also trapped ergosterol. After trapping of the ergosterol the silica gel was washed free of unreacted ergosterol and separated. Elution of the silica gel with an excess of triethyl amine in acetonitrile afforded the ergosterol-triazolinedione adduct. Treatment of this Diels-Alder adduct in refluxing THF containing excess LiAlH<sub>4</sub> liberated the ergosterol.

To date, although a number of attempts have been made, there still remains a 10 need for a method to produce oligonucleotides in large quantities, in continuous operations, at low cost and without laborious purification.

#### BRIEF SUMMARY OF THE INVENTION

The present invention is a method for the sequential solution phase synthesis 15 of oligonucleotides that increases reaction yields and allows for predictable scale-up. As opposed to traditional schemes in which the 3'-end of the growing oligonucleotide is bound to a solid support, the present invention is characterized by use of an anchor group attached to the 5'-end of the growing oligonucleotide that allows successfully coupled product to be separated from unreacted starting materials. In one embodiment, the anchor group also 20 serves as the 5'-OH protecting group and the coupling reaction occurs in solution. The successfully reacted oligomer will contain the protecting group, while the unreacted oligomer will not, and the materials can be partitioned based on the presence of the anchor/protecting group. In a preferred embodiment, the anchor group reacts covalently with a derivatized solid support, such as a resin, membrane or polymer.

25 Specifically, the invention provides a method for the solution phase synthesis of a wide variety of oligonucleotides and modified oligonucleotides comprising reaction of a 5'-protected monomer unit with the 5'-end of a growing oligonucleotide chain in solution. In an additional aspect of the invention, following reaction between the 5'-protected monomer unit and the growing oligonucleotide, the unreacted monomer may be oxidized to 30 form a charged species that may be easily partitioned from the remainder of the reaction medium. In the preferred embodiments of the invention the monomer units are

phosphoramidites, which upon activation and oxidation are converted to phosphates. The charged phosphate species can be easily partitioned from the remainder of the reaction medium.

In a preferred embodiment of the invention, the monomer unit consists of a

5 5'-protected phosphoramidite or H-phosphonate, wherein the protecting group is a substituted trityl group, levulinic acid group, or silyl ether group. In one embodiment, the unreacted oligonucleotide starting material (failure sequence) may be separated from the reacted oligonucleotide product based on the affinity of the protecting group for a chromatography resin. In a preferred embodiment, the unreacted oligonucleotide starting

10 material may be separated from the reacted oligonucleotide product based on the specific reaction of the protecting group with a derivatized solid support, such as a resin, membrane or polymer. In a preferred aspect of the invention the partitioning method to remove unreacted oligonucleotide serves to allow for isolation and reuse of the unreacted oligonucleotide and also will allow the reacted oligonucleotide to be deprotected in

15 preparation for the subsequent addition of the next 5'-protected monomer unit.

The method of this invention is not limited to phosphoramidite coupling chemistry, but is compatible with other coupling reactions such as H-phosphonate or phosphate triester coupling chemistry. This method also lends itself to automation and is ideally suited for the large scale manufacture of oligonucleotides with high efficiency. The

20 methods of the present invention can be extended to all sequential polymerization reactions and thus to the sequential synthesis of any polymer.

The present invention includes a method and apparatus to automatically separate the product from the unreacted 5'-protected monomer unit and the starting material. In one embodiment the apparatus is comprised of an extraction vessel and a chromatography

25 resin filtration chamber, which contains a solid support. Upon completion of a monomer addition reaction, the reaction mixture is pumped into the extraction chamber, extracted and then eluted through the solid support, which retains only the 5'-protected monomer unit. The product is then separated from the starting material by eluting through a solid support that retains only the product. In a second embodiment the chromatography resin filtration

30 chamber contains a solid support which covalently reacts with both the 5'-protected monomer unit and the product. The starting material is eluted from the solid support and

the monomer and product are then released from the solid support with a dilute acid. The product is then separated from the 5'-protected monomer unit by passage through an ultrafiltration membrane.

A material cost analysis reveals that the 5'-protected phosphoramidite is the 5 most costly reaction component in oligonucleotide synthesis. The cost of the remaining materials are trivial in comparison. Therefore, it would be desirable to make the monomer the limiting reagent. Furthermore, a particular intermediate oligonucleotide sequence which failed to add to the incoming monomer could serve as an intermediate in a subsequent synthesis. Using the method of this invention, verification of the sequence and composition 10 of oligonucleotide product becomes trivial. After every monomer addition cycle, a fully protected, neutral intermediate is obtained, which is easily analyzed by mass spectrometry without tedious sample preparation. Over the course of an oligonucleotide synthesis a library of analytical data for every sequential monomer addition can be obtained. Thus, product analysis becomes an integral part of the process.

15

#### BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 illustrates the reverse phase High Pressure Liquid Chromatography (HPLC) trace of the phosphoramidite coupling reaction mixture set forth in Example 1 prior to oxidation.

20

FIGURE 2 illustrates the reverse phase HPLC trace of the phosphoramidite coupling reaction set forth in Example 1 after oxidation. The post oxidation trace has been superimposed on the preoxidation trace of Figure 1.

25

FIGURE 3 illustrates the reverse phase HPLC traces of a mixture of oxidized phosphoramidite coupling reaction set forth in Example 1, both prior to and after being passed through a DEAE Sephadex® filter plug.

FIGURE 4 illustrates the reverse phase HPLC traces of the oxidized phosphoramidite coupling reaction set forth in Example 1, after being eluted through a C18 resin with water/acetonitrile and after treatment with acetic acid and elution with water/acetonitrile.

30

FIGURE 5 illustrates schematically an automated extraction and filtration system designed for use with the method of this invention.

FIGURE 6 illustrates the anion exchange HPLC trace of the 15 base oligonucleotide prepared in Example 7 using 3'-PEG anchored solution phase synthesis.

FIGURE 7 illustrates graphically the Diels-Alder capture data for the reaction of 5'-DHDT-T-[3',3']-T-OSiPDBT-5' with polystyrene maleimide resins containing 1.0 eq, 5 eq, 2.5 eq, 5 eq and 10 eq of maleimide.

FIGURE 8 illustrates schematically an automated extraction and filtration system designed for use with Diels-Alder product capture.

FIGURE 9 illustrates graphically the precipitation and centrifugation of PEG-precipitated by ethyl ether, isopropyl ether and N-butyl ether.

10 FIGURE 10 illustrates graphically the Diels-Alder reaction of diene substituted trityl alcohol 5'-O-(4,4'-di-3,5-hexadienoxytrityl)thymidine (5'-DHDT-dT) (31) with phenyltriazoline-dione (PTAD) (82) set forth in Example 17 (Scheme 22).

FIGURE 11 illustrates the use of a bis-TAD molecule in the PASS process. P is a solid support.

15 FIGURE 12 illustrates schematically a PASS cycle for a preferred embodiment of the instant invention.

FIGURES 13A-13E illustrate the HPLC traces of the reaction mixture for each step of PASS cycle described in Example 21.

20 FIGURE 14 illustrates the reversed phase HPLC trace of the T-T dimer prepared in Example 30.

FIGURE 15 illustrates the reversed phase HPLC trace of the T-T-T trimer prepared in Example 30.

25 FIGURE 16A illustrates the reverse phase HPLC trace of the reaction of DHDT fC 3'-OH (153) with TBDPSi-Cl after 65 minutes (Example 31). Coupling from top to bottom: 5'-DHDT 2'-fC 3'-OH (153) (13.8 minutes), TBDPSi-Cl (11.3 minutes), and 5'-DHDT 2'-fC 3'-TBDPSi (154) (19.8 minutes).

FIGURE 16B illustrates the reverse phase HPLC trace of the capture reaction of 5'-DHDT 2'-fC 3'-TBDPS (154) on a PTAD-PS resin. From top to bottom: 5'-DHDT 2'-fC 3'-TBDPSi (154) (19.8 minutes) and capture solution (10.4 and 11.3 minutes).

30 FIGURE 16C illustrates the reverse phase HPLC trace following the release and neutralization of compound 155. From top to bottom: aqueous solution 1 (1-2

minutes), aqueous solution 2 (no particular peaks) and 2'-fC 3'-TBDPS 5'-OH (155) (25.5 minutes).

FIGURE 16D illustrates the NMR of compound (155) 1'-cytidine (acetyl protected) 2'-flouro 3'-TBDPSi 5'-OH.

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#### DETAILED DESCRIPTION OF THE INVENTION

The present invention includes a method for the solution phase synthesis of oligonucleotides, referred to herein as Product Anchored Sequential Synthesis (PASS). Unlike traditional schemes where the 3'-end of the growing oligonucleotide is bound to a solid support, the present invention is characterized by utilization of an anchor group attached to the 5'-end of the growing oligonucleotide product that allows successfully coupled product to be separated from unreacted starting materials. In a preferred embodiment the anchor group also serves as the 5'-OH protecting group. The successfully reacted oligonucleotide product will contain the protecting group, while the unreacted oligonucleotide starting material will not, and the product can be partitioned away from the starting material based on the presence of the blocking/protecting group. Unreacted starting material is recovered and can be reused in a subsequent synthesis batch of the same oligonucleotide. Thus, in contrast to conventional solid phase synthesis, the improved method for oligonucleotide synthesis described herein does not employ a solid support for anchoring of the 3'-end of the growing oligonucleotide chain.

Specifically, the invention provides a method for the solution phase synthesis of a wide variety of oligonucleotides and modified oligonucleotides comprising reaction of a 5'-protected monomer unit with the 5'-end of a growing oligonucleotide chain in solution. Performing these reactions in solution, rather than on solid supports, provides for better reaction kinetics. In an additional aspect of the invention, following reaction between the 5'-protected monomer unit and the growing oligonucleotide, the unreacted 5'-protected monomer unit may be activated and oxidized to form a charged species that may be easily partitioned from the remainder of the reaction medium. In the preferred embodiments of the invention the monomer units are phosphoramidites, that upon oxidation can be easily converted to phosphate diesters. The charged phosphate species can be easily partitioned

from the remainder of the reaction medium. Additionally, in a preferred embodiment the oxidation may be performed *in situ*.

When using a H-phosphonate as the 5'-protected monomer unit, which is a charged species (Example 2, Scheme 5), the oxidization of H-phosphonate is deferred until 5 after the addition of the final monomer. The charged H-phosphonate monomers produce neutral H-phosphonate diester products after coupling, and the charged monomer species are readily removed by anion exchange filtration or extraction. In addition, the recovered H-phosphonate monomers are reusable.

The 5'-protecting group that is utilized can be selected from any class of 10 chemical functionalities that meets the basic requirements of the invention. The protecting group must be of a type that can be used to differentiate the product of the reaction from the remainder of the reaction mixture in order to effect a separation. Preferably, the protecting group will have a strong affinity for or a reactivity with a particular phase or solid support and it must be easily cleaved or removed from the phase or solid support with high 15 selectivity. The oligonucleotide product may be separated from unreacted oligonucleotide starting material using standard methods known to those skilled in the art including, but not limited to, centrifugation, separation on a resin, silica gel based separation, separation based on affinity for a metal, separation based on a magnetic force or electromagnetic force or separation based upon covalent attachment to a suitable solid support.

20 In a preferred aspect of the invention the partitioning method to remove unreacted oligonucleotide starting material serves to both allow for the isolation for reuse of the unreacted oligonucleotide and also will result in a resin-bound oligonucleotide product which is easily deprotected in preparation for the subsequent addition of the next 5'-protected monomer unit. Most preferably, the protecting group will covalently react with a 25 derivatized solid support, such as a resin, membrane or polymer, to give a covalently anchored protecting group which may easily be cleaved from the oligonucleotide with high selectivity.

In the most preferred embodiment of the invention, the monomer unit 30 consists of a 5'-protected phosphoramidite or H-phosphonate, wherein the protecting group is a substituted trityl group, levulinic acid group or silyl ether group. The preferred substitution on the protecting group is a diene functionality, which can react, via a Diels-

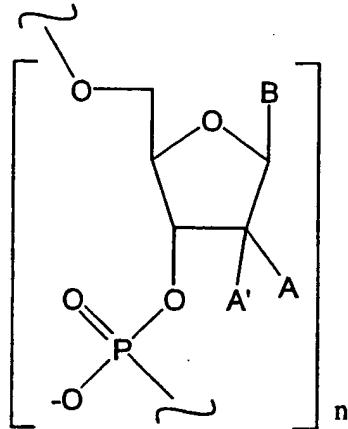
Alder reaction, with a solid support, such as a resin, membrane or polymer that has been derivatized with a dienophile, preferably a 1,2,4-triazoline-3,5-dione (TAD). In this embodiment, the unreacted oligonucleotide starting material is separated from the reacted nucleotide product based on the selective or specific covalent reaction of the 5'-protecting group with a derivatized resin.

5        Certain terms used to describe the invention herein are defined as follows:  
 "Nucleoside" means either a deoxyribonucleoside or a ribonucleoside or any chemical modifications thereof. Modifications of the nucleosides include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position 10      purine modifications, modifications at cytosine exocyclic amines, substitution of 5-bromo-uracil, and the like.

15      "Oligonucleotide" refers to either DNA or RNA or any chemical modifications thereof. The oligonucleotides synthesized by the method of this invention are depicted generally as follows:

15

20



25      where  $n = 1 - 1,000$ , A and A' are 2'-sugar substituents as defined below and B is a nucleobase as defined below.

30      A "solid support" as used herein refers to a resin, membrane, phase, polymer, polymer precursor, or soluble polymer that can undergo phase transition. A solid support also refers to a resin, membrane, phase, polymer, polymer precursor, or soluble polymer that has been derivatized with a diene, dienophile, 1,3 dipole or a D group, as defined below. The term resin and solid support are used interchangeably and one of ordinary skill in the art will recognize what is intended by the term resin. Examples of solid supports include, but

are not limited to, organic polymers, crosslinked organic polymers, maleimide derivatized polystyrene, polystyrene derivatized with D groups, as defined below, dienophile or diene derivatized polystyrene, Tentagel™ derivatized with D groups, as defined below, dienophile or diene derivatized Tentagel™, 1,2,4-triazoline-3,5-dione (TAD) derivatized resins, 5 phenyltriazoline-dione (PTDA) derivatized resins, diene or dieonphile derivatized amino-functionalized resins, such as aminomethyl polystyrene, aminopropyl silica gel, aminopropyl CPG, NovaSyn™ TG amino resin HL, and ArgoGel™, amino-functionalized resins derivatized with D groups, as defined below, 4-(1,2,4-triazoline-3,5-dione)benzoic acid derivatized amino-functionalized resins, dienophile or diene derivatized ultrafiltration 10 membranes, dienophile or diene derivatized polyethylene glycol, diene or dienophile derivatized inorganic oxides, such as silica gel, alumina, controlled pore glass and zeolites, other dienophile or diene derivatized polymers, hydrophobic reverse phase resins, such as C2 to C18 polystyrene, cellulose, methacrylate, dendrimer, thiopropyl Sepharose (Pharmacia Biotech), mercurated resin, agarose adipic acid hydrazide (Pharmacia Biotech), amino- 15 functionalized cellulose beads or avidin resin.

A "diene" is defined as a molecule bearing two adjacent double bonds, where the atoms forming these double bonds can be carbon or a heteroatom, which can undergo a [2+4] cycloaddition reaction with a dienophile.

A "dienophile" is defined as a molecule bearing an alkene group, or a double 20 between a carbon and a heteroatom, or a double bond between two heteroatoms, which can undergo a [2+4] cycloaddition reaction with a suitable diene.

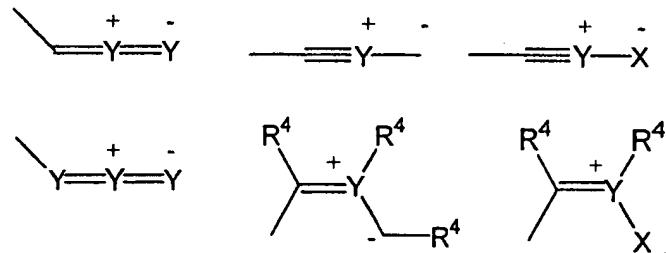
The dienophile can be any group, including but not limited to, a substituted 25 or unsubstituted alkene, or a substituted or unsubstituted alkyne. Typically, the dienophile is a substituted alkene of the formula C=C-Z or Z'-C=C-Z, wherein Z and Z' are electron withdrawing groups independently selected from CHO, COR, COOH, COCl, COAr, CN, NO<sub>2</sub>, Ar, CH<sub>2</sub>OH, CH<sub>2</sub>Cl, CH<sub>2</sub>NH<sub>2</sub>, CH<sub>2</sub>CN, CH<sub>2</sub>COOH, halogen, or C=C. Other dienophiles include, but are not limited to thiocarbonyl compounds, including thioaldehydes, thioesters, thioketones, thiocarbamates, thiocarbonates and thioamides and nitroso compounds.

30 A "dienophile derivatized solid support" refers to a solid support that has been functionalized with a dienophile and a "diene derivatized solid support" refers to a

solid support that has been functionalized with a diene. Preferred solid supports are inorganic oxides selected from the group consisting of silica, alumina, zeolites, controlled pore glass, that have hydroxyl groups that are capable of being functionalized, or organic supports such as polystyrene, as illustrated in Schemes 13 and 14. In a preferred embodiment the dienophile is maleimide and the diene is 3,5-hexadiene. In the most preferred embodiment the dienophile is a 1,2,4-triazoline-3,5-dione (TAD).

A "[3+2] dipole" is defined as any moiety capable of undergoing a 3+2 cycloaddition reaction with a substituted or unsubstituted alkene, or a substituted or unsubstituted alkyne. Examples of [3+2] dipoles include, but are not limited to compounds of the formula:

15

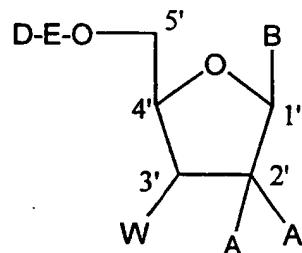


wherein Y, R<sup>4</sup> and X are as defined below.

The "5'-protected monomer units" of this invention are generally depicted as follows including the conventional numbering for the ribose ring:

20

25



B is a nucleobase;

A and A' are 2'-sugar substituents;

W is independently selected from the group consisting of a phosphoramidite, H-

30 phosphonate, phosphotriester, phosphoramidate, protected oligonucleotide and methyl-phosphonate; and

D-E is an alcohol protecting group(s) which serves as an anchor for partitioning the successfully reacted oligonucleotide product away from the unreacted oligonucleotide starting material.

Other obvious substitutions for the substituents described above are also 5 included within the scope of this invention, which is not limited to the specific, but rather the generalized formula of reaction.

In a preferred embodiment of the invention:

W is a phosphoramidite or H-phosphonate;

A and A' are independently selected from the group consisting of H, <sup>2</sup>H, <sup>3</sup>H, Cl, F, OH, 10 NHOR<sup>1</sup>, NHOR<sup>3</sup>, NHNHR<sup>3</sup>, NHR<sup>3</sup>, =NH, CHCN, CHCl<sub>2</sub>, SH, SR<sup>3</sup>, CFH<sub>2</sub>, CF<sub>2</sub>H, CR<sup>2</sup>Br, - (OCH<sub>2</sub>CH<sub>2</sub>)<sub>n</sub>OCH<sub>3</sub>, OR<sup>4</sup> and imidazole (*see* United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of 2' Modified Pyrimidines Intramolecular Nucleophilic Displacement," which is incorporated herein by reference);

15 R<sup>1</sup> is selected from the group consisting of H and an alcohol protecting group; R<sup>2</sup> is selected from the group consisting of =O, =S, H, OH, CCl<sub>3</sub>, CF<sub>3</sub>, halide, optionally substituted C<sub>1</sub>-C<sub>20</sub> alkyl (including cyclic, straight chain, and branched), alkenyl, aryl, C<sub>1</sub>-C<sub>20</sub> acyl, benzoyl, OR<sup>4</sup> and esters;

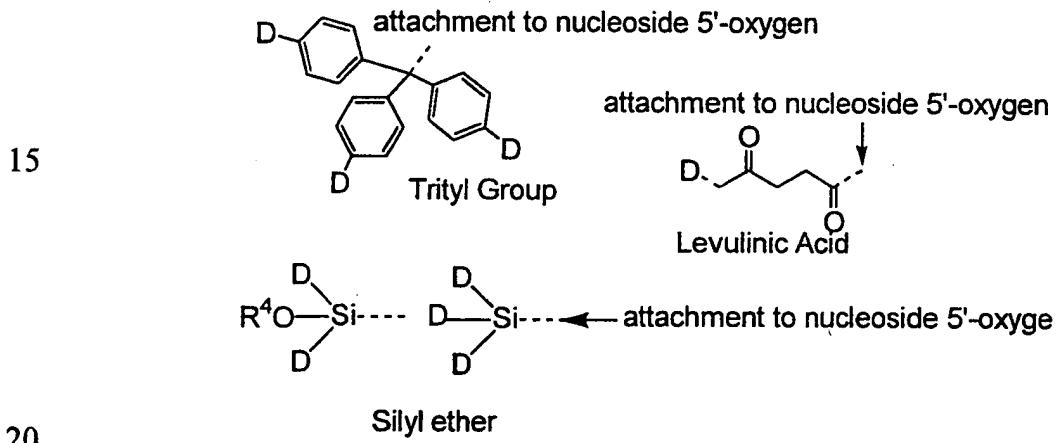
R<sup>3</sup> is selected from the group consisting of R<sup>2</sup>, R<sup>4</sup>, CN, C(O)NH<sub>2</sub>, C(S)NH<sub>2</sub>, C(O)CF<sub>3</sub>, 20 SO<sub>2</sub>R<sup>4</sup>, amino acid, peptide and mixtures thereof;

R<sup>4</sup> is selected from the group consisting of an optionally substituted hydrocarbon (C<sub>1</sub>-C<sub>20</sub> alkyl, C<sub>2</sub>-C<sub>20</sub> alkenyl, C<sub>2</sub>-C<sub>20</sub> alkynyl), an optionally substituted heterocycle, t-butyldimethylsilyl ether, triisopropylsilyl ether, nucleoside, carbohydrate, fluorescent label and phosphate; most preferably A is selected from the group consisting of H, OH, NH<sub>2</sub>, Cl, 25 F, NHOR<sup>3</sup>, OR<sup>4</sup>, OSiR<sup>4</sup><sub>3</sub>. (*See* United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of 2' Modified Pyrimidines Intramolecular Nucleophilic Displacement," filed June 22, 1994);

D-E can be any group that enables the partitioning of the "growing oligonucleotide chain" or "oligonucleotide product" away from unwanted side products and starting materials. The 30 partitioning can be done by any suitable method, including but not limited to, silica gel based chromatography, centrifugation, or any other means known by those in the art for

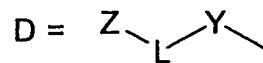
partitioning materials. The preferred method for partitioning is by binding to a resin. The most preferred method for partitioning is by covalent reaction between D and a derivatized solid support, such as a derivatized resin, polymer, or membrane. The protecting group D-E, therefore, is preferably designed such that D has a strong affinity for a particular resin or phase, and E is designed such that the 5'-oxygen-E bond is easily cleaved with high selectivity. In cases where E shows high affinity for a resin or phase, D may be omitted. Most preferably the protecting group D-E is designed such that D can selectively or specifically form a covalent bond to a particular derivatized resin, polymer, or membrane. This covalent bond between D and a resin can be reversible.

10 E includes, but is not limited to, the trityl group or the levulinic acid group or a silyl ether group, as depicted below.



D includes, but is not limited to, groups independently selected from H, OR<sup>4</sup>, an alkyl or substituted alkyl group bearing a conjugated diene unit, an alkoxy or substituted alkoxy group bearing a conjugated diene unit, CH<sub>2</sub>=CHCH=CHCH<sub>2</sub>CH<sub>2</sub>O-, CH<sub>2</sub>=CHCH=CHCH<sub>2</sub>CH<sub>2</sub>O-, an alkenyl or substituted alkenyl group, maleimide substituted alkoxy groups, dienophile substituted alkoxy groups, alkoxy groups, an alkylamino or substituted alkylamino group bearing a conjugated diene unit, maleimide substituted alkylamino groups or substituted alkylamino groups, an alkylamino group or substituted alkylamino group bearing a dienophile moiety, a solid support, a 1, 3-dipolar group, a substituent capable of undergoing ring-opening metathesis polymerization, such as a 7-oxanorbornene containing substituent, disulfides, aldehydes, and metal chelators, silyl ethers bearing dienophile or diene units, some examples of which are depicted below. The

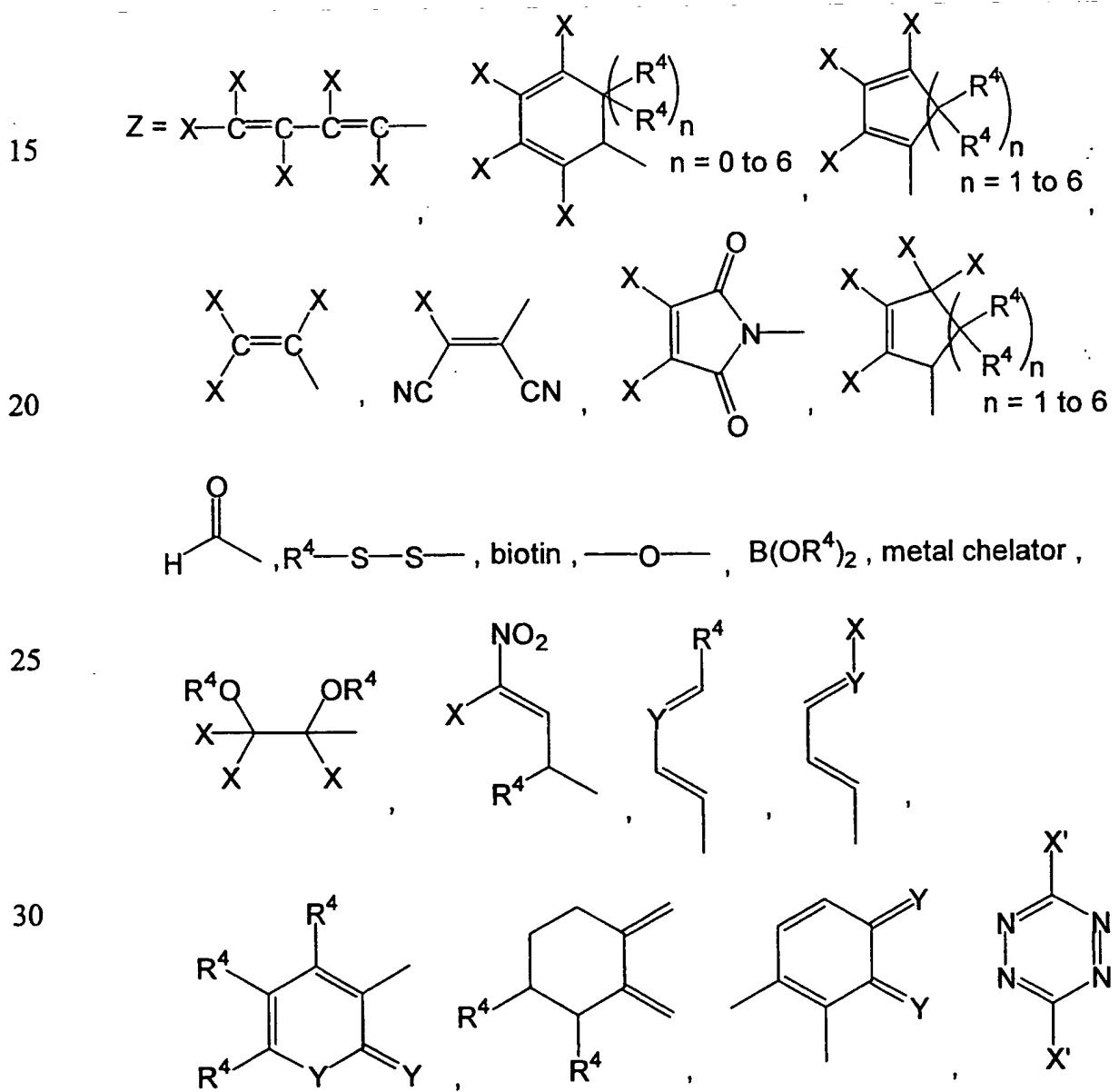
alkyl groups on the above listed substituents can have between 1-50 carbons, preferably 1-30 carbons.

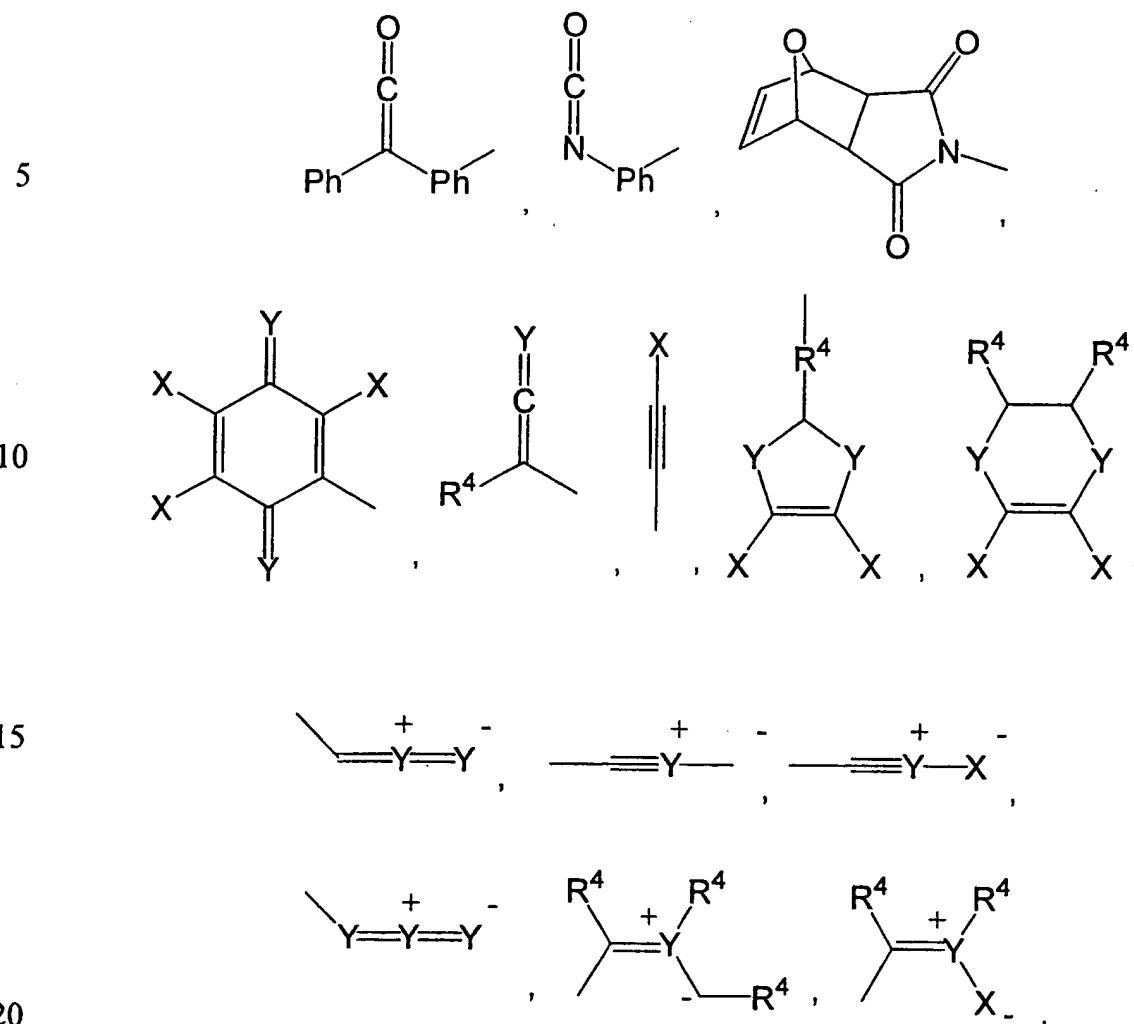


5 Y = O, NH, S, P(H)(OR<sup>4</sup>), P(OR<sup>4</sup>)<sub>2</sub>, POH(O)(OR<sup>4</sup>), NH(CO), (CO)NH, O(CO)  
(CO)O, NH(CO)NH, NH(CO)O, O(CO)NH, NH(CS)NH, NH(CS)O, O(CS)N  
omitted, SO, SO<sub>2</sub>

L = a linking group

10 X = electron withdrawing group or electron donating group





For the purposes of this invention "nucleobase" will have the following definition. A nucleobase is a purine or a pyrimidine base. Nucleobase includes all purines and pyrimidines currently known to those skilled in the art or any chemical modifications thereof. The purines are attached to the ribose ring through the nitrogen in the 9 position of the purine ring and the pyrimidines are attached to the ribose ring through the nitrogen in the 1 position of the pyrimidine ring. The pyrimidine can be modified at the 5- or 6- position of the pyrimidine ring and the purine can be modified at positions 2-, 6- or 8- of the purine ring. Certain modifications are described in copending United States Patent Applications Serial Nos. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2' Modified Pyrimidines Intramolecular Nucleophilic Displacement" and

08/458,421, filed June 2, 1994, entitled "Palladium Catalyzed Nucleoside Modifications - Methods Using Nucleophiles and Carbon Monoxide" and United States Patent No. 5,428,149, entitled "Method for Palladium Catalyzed Carbon-Carbon Coupling and Productions," which are herein incorporated by reference. More specifically a nucleobase 5 includes, but is not limited to, uracil, cytosine, N4-protected cytosine, 4-thiouracil, isocytosine, 5-methyluracil (thymine), 5-substituted uracils, adenine, N6-protected adenine, guanine, N2-protected guanine 2,6-diaminopurine, halogenated purines as well as heterocycles meant to mimic the purine or pyrimidine ring, such as imidazole.

"Starting material" as used herein refers to the compound that is reacted with 10 the 5'-protected monomer unit during each cycle of PASS to produce an oligomer that has been extended by one or more nucleotides. The starting material can be designed to produce a [5',3'] linkage between nucleotides or a [3',3'] linkage between nucleotides, depending on the desired oligonucleotide product. In the first instance the starting material is a 5'- deprotected otherwise protected oligonucleotide of length n, in the second case the starting 15 material is a 3'-deprotected otherwise protected oligonucleotide of length n. Typically the starting material is a 5'-deprotected otherwise protected oligonucleotide of length n, wherein n is an integer from 1-1000. The starting material is 2', 3'-protected by protecting groups, such as base labile groups, that are compatible with the reaction of the 5'-protected monomer units with the starting material and with 5'-deprotection reactions. Additionally, 20 because the PASS process consists of the controlled and sequential polymerization of an oligonucleotide, the starting material of one PASS cycle is typically the deprotected product from the previous PASS cycle. Because the PASS process does not require that the 3'- terminal nucleotide be anchored to a solid support, the starting material can include non-nucleoside modifications. Non-nucleoside modifications can be introduced to the 3'- 25 terminus which would not ordinarily be possible by solid phase synthesis. Non-nucleoside modifications to the 3'-terminus of the starting material include, but are not limited to, the use of polyethylene glycol mono-methylether (molecular weight 5,000 to 100,000) (PEG) or other high molecular weight non-immunogenic units as the 3'-terminal monomer for preparation of oligonucleotides with improved pharmacokinetic properties.

30 "Product" as used herein refers to an oligonucleotide that is produced by the covalent reaction of the 5'-protected monomer unit with the starting material during each

PASS cycle. As stated above, if the starting material is a 5'-deprotected oligonucleotide of length n and the 5'-monomer unit is a single nucleotide, the product of the reaction will be a 5'-protected oligonucleotide of length n+1. If the 5'-protected monomer unit is an oligonucleotide block of length m the product of the reaction will be a 5-protected 5 oligonucleotide of length n+m. The product from a particular PASS cycle is then 5'- deprotected and becomes the starting material for the next cycle.

A "failure sequence" refers to the starting material from a particular PASS cycle that fails to react with the 5'-protected monomer unit during that cycle.

"Growing oligonucleotide chain" refers to either a 5'-deprotected 10 oligonucleotide chain or a 5'-protected oligonucleotide chain that has been prepared by the sequential addition of nucleotides (N) beginning with the 3'-terminal nucleotide of the desired nucleotide using the method of this invention. After each reaction cycle of the PASS process the growing oligonucleotide increases in length by at least one oligonucleotide, and becomes the starting material for the next reaction cycle. As used 15 herein the term can refer to either starting material or product and one of ordinary skill in the art will recognize what is intended by the term in a particular context.

Scheme 2 generally illustrates the method of this invention. A 5'-protected monomer unit, such as phosphoramidite 7, is added to a starting material 8 in solution, in the presence of an activator, such as tetrazole or preferably 4,5-dicyanoimidazole (DCI) (see 20 United States Patent Application Serial No. 08/730,556, filed October 15, 1996, entitled "Improved Coupling Activators for Oligonucleotide Synthesis"), to yield a product 9 to which one nucleotide has been added via a phosphite triester linkage. As depicted in this figure the starting material 8 is a 5'-deprotected otherwise protected oligonucleotide of length n, wherein n is an integer between 1 and 1000, and the product is a 5'-protected 25 oligonucleotide of length n+1. The 5'-deprotected oligonucleotide starting material 8 is not anchored to a solid support, but rather, using standard methods, is simply 2', 3'-protected by protecting groups, such as base labile groups, that are compatible with the reaction of the 5'- protected monomer units with the starting material and with 5'-deprotection reactions. The elimination of 3'-anchoring to a solid support enhances the scope of the 3'-modifications that 30 can be incorporated into oligonucleotides. Additionally, the 3'-terminal nucleotide no longer has the requirement of bearing the hydroxyl substituent needed for support

anchoring. Thus, modifications can be introduced to the 3'-terminus which are not possible by solid phase synthesis. This includes, but is not limited to, the use of polyethylene glycol mono-methylether (molecular weight 5,000 to 100,000) or other high molecular weight non-immunogenic units as the 3'-terminal monomer for preparation of oligonucleotides with 5 improved pharmacokinetic properties. (See United States Patent Application Serial No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes," which is incorporated herein by reference).

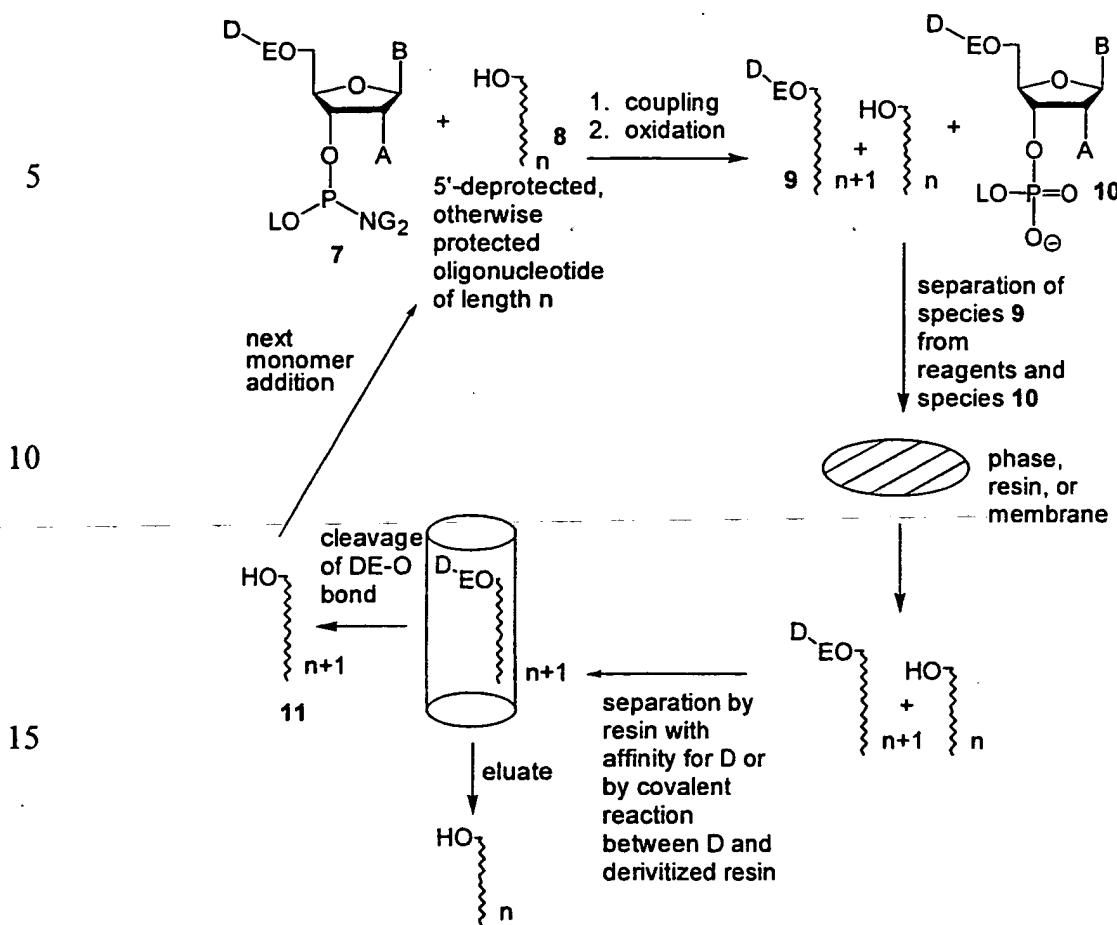
After completion of the reaction between the 5'-protected monomer unit 7 and starting material 8, the reaction mixture contains three species: unreacted 5'-protected 10 monomer unit 7, unreacted starting material 8, and the product of the reaction, compound 9, which is a 5'-protected oligonucleotide of length n+1. As discussed above any of the starting material 8 (a 5'-deprotected oligonucleotide of length n) which fails to react with the 5'-protected monomer unit 7 is referred to as the failure sequence, as this sequence was not extended. The product of the reaction, compound 9, is a 5'-protected oligonucleotide chain 15 extended by one nucleotide (length n+1), by the covalent reaction of the 5'-hydroxy group of starting material 8, an oligonucleotide of length n with the 3'-phosphoramidite group of the 5'-protected monomer unit 7. The product, compound 9, is the major component and the 5'-protected monomer unit 7 and the starting material 8 that did not react are present only in minor amounts.

20 At this stage of the process the unreacted 5'-protected monomer unit may optionally be removed from the reaction mixture, both to purify the materials and to recover the monomer starting material. According to this embodiment, non-reacted monomer is reacted to form an easily removable ionic species. Oxidation of the phosphite triester to phosphate triester may be carried out in the same reaction flask simply by addition of an 25 oxidizing agent. *In situ* oxidation gives the desired oligonucleotide product 9, the phosphate salt 10 of monomer 7, as well as unreacted oligonucleotide starting material 8. The monomer phosphate salt 10 is the only free salt in the reaction mixture and thus is easily removed by techniques known to those in the art, including but not limited to, filtration through an anion exchange resin or membrane or extraction with an aqueous phase. In an 30 alternate variation of this embodiment of the invention, the 3'-terminal monomer is a polyethylene glycol mono-methylether of molecular weight 5,000 to 100,000, preferably

20,000. In this case, a simple molecular weight cut-off membrane can be used to remove monomer **10**.

After the unreacted monomer has been removed from the reaction mixture, the remaining filtrate may then be partitioned in any manner suitable to separate the "oligonucleotide product" from the "failure sequence." In one embodiment, the filtrate is applied to a material designed to interact selectively or specifically with the 5'-protecting group (D-E), such as a reverse phase resin. The product is captured or retained on the solid support by affinity of the 5'-protecting group constituent D with the resin. In a preferred embodiment, the filtrate is applied to a material designed to covalently react with the 5'-protecting group (D-E), such as a dienophile derivatized resin where D contains a diene unit. The product is captured or retained on the solid support by covalent reaction of the 5'-protecting group constituent D with the resin. The unreacted oligonucleotide starting material **8**, which does not carry the 5'-protecting group D, is washed away. The unreacted starting material may be isolated and stored to be used as an intermediate in a subsequent synthesis. The retained oligonucleotide product **9** is then released from the resin according to well known procedures. In certain embodiments, the oligonucleotide product is released by cleavage of the bond between the 5'-oxygen and the protecting group D-E. For example, when the 5'-protecting group is a trityl derivative, a reagent such as dilute dichloroacetic acid (DCA) may be used to cleave the trityl group, thereby releasing the oligonucleotide coupling product. The liberated 5'-deprotected oligonucleotide coupling product **11** can then be used as the starting material in an additional coupling reaction.

## SCHEME 2



20 It is not a requirement of the present invention that the steps in the monomer addition cycle depicted in Scheme 2 occur in the exact sequence described above. Alternatively, coupling and oxidation *in situ* can be followed by covalent or affinity capture of the product and of monomer 10 on a resin. Subsequent cleavage of the 5'-protecting group liberates both the product and the monomer. At this stage an extraction or membrane-based filtration easily

25 removes the unwanted monomer byproduct.

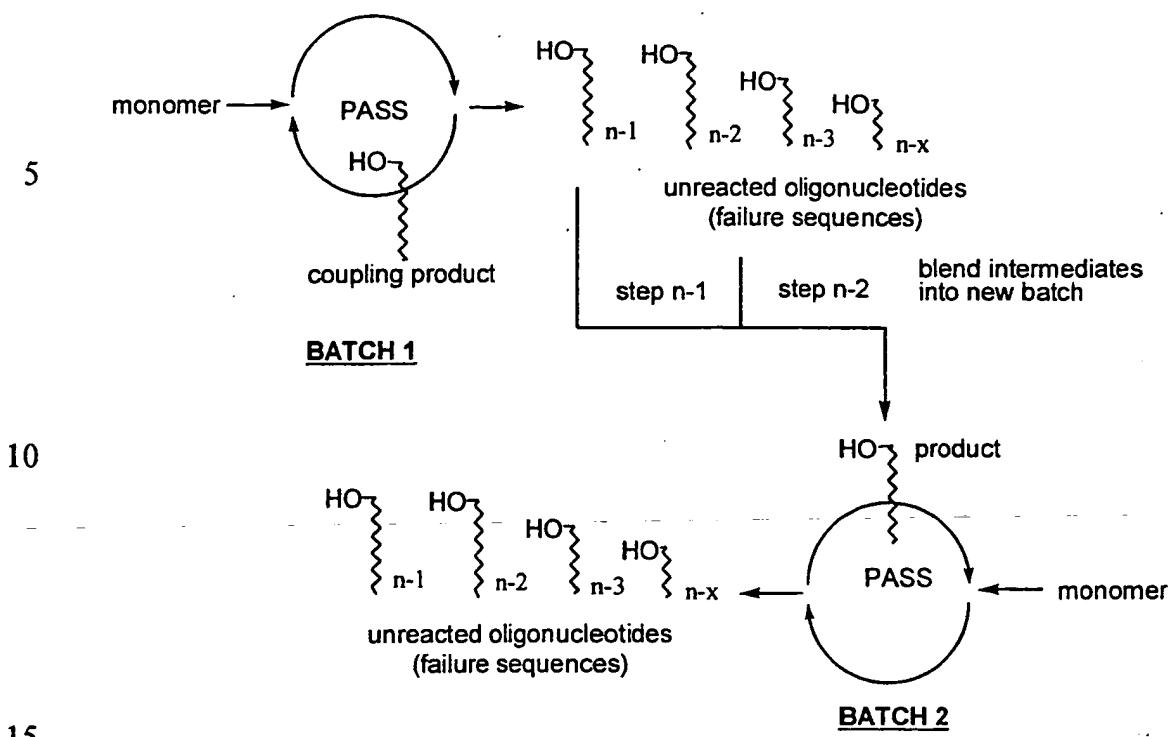
Utilization of the 5'-protecting group for anchoring of the oligonucleotide product allows for the possibility of using a wide variety of 3'-terminal modifications. These can be groups designed to facilitate separation of the product of the reaction from the 5'-protected monomer unit, such as a polymer of sufficient molecular weight to exploit

30 molecular weight cut-off membranes for this separation, or a metal chelator to effect selective precipitation of the product. In such a case these groups contain a cleavable linker

between the 3'-terminus of the oligonucleotide and the modifying group, such as a succinate linker. Alternatively, non-nucleoside 3'-terminal substituents may enhance pharmacokinetic properties of oligonucleotide products, such as a polyethylene glycol mono-methylether or a distearyl glycerol. (See United States Patent Application Serial No. 08/434,465, filed May 5 4, 1995, entitled "Nucleic Acid Ligand Complexes," which is incorporated herein by reference). The 3'-terminal monomer may also serve as a detector for diagnostic applications of oligonucleotides, such as a chelator designed to retain Tc99m for *in vivo* imaging. (See Patent Application No. WO 96/02274, published February 1, 1996, entitled "Conjugates Made of Metal Complexes and Oligonucleotides, Agents Containing the 10 Conjugates, Their Use in Radiodiagnosis as well as Process for Their Production," which is incorporated herein by reference). In conventional solid phase oligonucleotide synthesis the 3'-terminus is not accessible for introduction of such constituents since it is utilized to anchor the growing chain to the solid support.

In contrast to the conventional solid phase synthesis process, the 15 oligonucleotide product is preferably separated from unreacted starting material each time a new coupling reaction is performed. Thus, the final oligonucleotide product is obtained in essentially pure form and the cumbersome removal of highly homologous failure sequences is eliminated. Additionally, because the reaction is performed in the solution phase, the yields of the reaction of the monomer with the oligonucleotide starting material are also 20 significantly increased. Furthermore, a capping step becomes superfluous in this scheme, since only successful oligonucleotide coupling products enter the next step of the process. The elimination of the capping step amounts to another efficiency gain compared to the conventional process. The oligonucleotide starting material that failed to undergo reaction with the 5'-protected monomer unit (failure sequence) is instead isolated and may be reused. 25 Each time a failure sequence is reisolated during a PASS iteration, it can be blended into the starting material at the same step or iteration in a subsequent synthesis of the same oligomer, or of an oligomer that shares the same 3'-terminal fragment. (See Scheme 3). Failure sequences, therefore, become useful sequential building blocks for the subsequent manufacture of oligonucleotides. This not only increases the efficiency of the process, it 30 also dramatically increases the purity of the final crude product. It further allows using the monomer as the limiting reagent and thus, dramatically increases process efficiency.

## SCHEME 3



The outlined synthetic scheme, which exploits the 5'-protecting group as the anchor for separation of product from starting materials and allows failure sequences to become intermediates for subsequent syntheses, is not limited to phosphoramidite coupling chemistry. It is compatible with other coupling reactions, such as, H-phosphonate or 20 phosphate triester coupling chemistry. (See Gaffney and Jones (1988) *Tetrahedron Lett.* 29:2619-2622). This scheme also lends itself to automation of oligonucleotide synthesis and is ideally suited for the large scale manufacture of oligonucleotides with high efficiency.

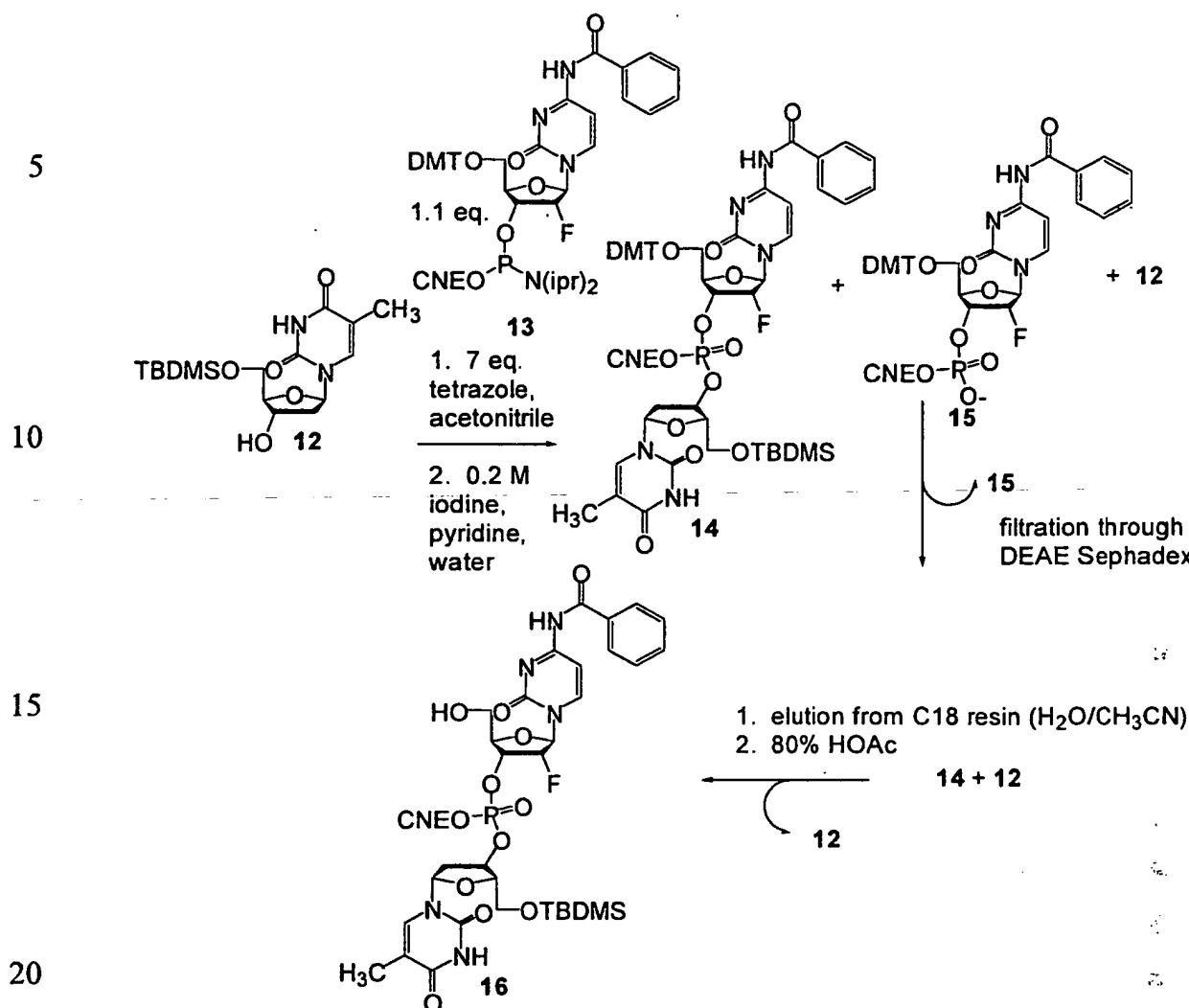
Aspects of the technology described here have applications beyond the PASS 25 synthesis process. For instance, the covalent capture of desired or unwanted species in oligonucleotide synthesis can also be applied to a high resolution, single-step purification method in conventional solid phase or solution phase processes. If only the terminal monomer bears a diene modified trityl group at its 5'-terminus, then selective anchoring of the full length product on a dienophile derivatized resin or membrane removes all major 30 failure sequences from the crude mixture. (See Scheme 1). In another application, if a capping reagent is used which contains a moiety suitable for covalent capture (all D groups

described above apply), such as a diene-modified acetic anhydride (or generally a D-modified acetic anhydride) or diene modified silyl chloride, such as 3,5-hexadienoxyacetic anhydride or tri-(3,5-hexadienoxy)silyl chloride, then all capped failure sequences can be removed from a crude oligonucleotide batch either after every monomer addition (in 5 solution phase oligonucleotide synthesis processes) or after cleavage of the crude oligonucleotide from the solid support (in conventional solid phase oligonucleotide synthesis processes). In yet another application, the reaction of a diene modified trityl group with a dienophile modified resin allows facile preparation of cation exchange resins.

A dimer of 2'-fluoropyrimidine modified RNA oligonucleotides is assembled 10 by the PASS process in Example 1 (Scheme 4). In the first reaction phosphoramidite coupling chemistry is employed to form a 3',3'-phosphodiester linkage. Oligonucleotides are often protected against 3'- to 5'-exonucleolytic degradation by incorporation of a 3',3'-phosphodiester linkage at the 3'-terminus. After coupling, the reaction mixture is oxidized *in situ* to produce unreacted thymidine starting material 12, oxidized amidite monomer 15, 15 and oxidized dimer product 14.

The oxidized amidite monomer 15 is removed by filtering the reaction mixture through a bed of diethylaminoethylene (DEAE) Sephadex®. HPLC analysis of the filtrate indicates that the oxidized amidite monomer 15 has been retained by the DEAE Sephadex® as shown in Figure 3. The filtrate, which contains the oxidized dimer product 14 20 and the unreacted thymidine starting material 12, is concentrated and redissolved in 60% acetonitrile/water and loaded onto a C18 filter plug. The resin is washed with 70% water/acetonitrile followed by 50% water/acetonitrile to fully elute the unreacted thymidine starting material 12. The resin, which now contains only the tritylated dimer product 14 is then washed with water, followed by treatment with 80% acetic acid/water to effect 25 detritylation. The resin is then washed with 50% acetonitrile/water, which elutes the final product 16, while retaining the trityl species (Figure 4).

## SCHEME 4



Example 2 (Scheme 5) illustrates the method of this invention, wherein the 5'-protected monomer unit is an H-phosphonate, rather than a phosphoramidite. In this example an H-phosphonate thymidine trimer bearing a 3',3'-internucleotidic linkage at the 3'-terminus (T-T-[3',3']-T trimer) 20 is prepared. The efficiency of the liquid phase coupling reaction was so high, that no unreacted 3'-terminal fragment 19 was detected. Thus, the reverse phase step is used only to cleave and separate the trityl group from the product.

Example 3 (Scheme 6) describes the synthesis of a phosphoramidite monomer containing 5'-O-(4,4'-dioctadecyloxytrityl) (DOT) as the 5'-protecting group (D-E).

Example 4 illustrates the ability to separate the coupling product from the unreacted oligonucleotide starting material (failure sequence) based upon the selective or 5 specific interaction of the 5'-protecting group (D-E) with a particular resin or phase. In this example, the mobility of 4,4'-dioctadecyltriphenylmethanol (DOT) 23 on a C18 reverse phase resin is compared to that of 4-decyloxy-4'-methoxytritanol and dimethoxytritanol (DMT) (see Table 1). The strong interaction of the DOT group with C18 resin in organic solvents, such as methanol ( $R_f=0$ ) and acetonitrile ( $R_f=0$ ) enables the one-step separation of 10 product from starting material by loading the mixture onto C18 resin and washing the unreacted starting material away with an organic solvent. The coupled product can then be eluted from the chamber by cleavage of the trityl protecting group with a haloacetic acid in an organic solvent. The trityl group is retained on the resin.

Example 5 describes the assembly of a hexamer oligonucleotide (5'-HO-T-T-15 A-C-T-[3',3']-T) in solution using an anion exchange medium to remove the excess monomer and C18 reverse phase resin to selectively capture the 5'-DMT protected product while not retaining the failure sequence. As can be seen in Example 5, each monomer addition is accomplished in two steps. In the first step phosphoramidite coupling chemistry is employed to couple the 5'-protected monomer unit to the starting material. After 20 coupling, the reaction mixture is oxidized *in situ* to produce unreacted starting material (failure sequence), oxidized amidite monomer, and oxidized product. The oxidized amidite monomer is removed by filtering the reaction mixture through an anion exchange medium, such as, DEAE Sephadex®.

In the second step the filtrate, which contains the oxidized product and the 25 unreacted starting material (failure sequence), is treated with a dilute acid to effect detritylation. Examples of dilute acids which can be used include, but are not limited to, dilute mineral acids, dilute trichloroacetic acid, dilute dichloroacetic acid (DCA), Lewis acids, such as,  $ZnBr_2$ , nitromethane, tosic acid and perchloric acid. The mixture is then separated by chromatography. Alternatively, the mixture of product and unreacted starting 30 material is first separated using a reverse phase resin followed by detritylation to release the detritylated product from the resin. The analytical data provided in Example 5 shows that

the PASS process produces essentially pure oligonucleotide intermediates at every iteration with minimal consumption of cost-limiting monomer.

Example 6 (Figure 5) illustrates schematically an automated extraction/filtration system 110 designed for use with the method of this invention, to separate the unreacted 5'-protected monomer unit from the remainder of the reaction mixture. As stated above, the method of this invention lends itself to automation and is thus ideally suited for large scale manufacture of oligonucleotides. The automated extraction/filtration system 110 has two centers: an extraction vessel 112 and a chromatography resin filtration chamber 114. The extraction vessel is in fluid communication with the chromatography resin chamber by a tube 118. A first three way valve 120 controls the flow of the contents from the extraction vessel 112 into the chromatography chamber 114. A second valve 122 controls the addition of solvents into chamber 114. A third valve 124 controls the collection of effluent out of chamber 114. All three valves are electronically coupled to a controller 126, that provides signals that actuate all three valves 120, 122, and 124 between their various flow positions.

Extraction vessel 112 is equipped with two inlet ports, 128 and 130, a stirrer 132, and an outlet port 134. The reaction mixture is pumped into the extraction vessel 112 through inlet port 128 and an extraction solvent, such as  $\text{CH}_2\text{Cl}_2$ , and an aqueous buffer are pumped into the extraction vessel through inlet port 130. The mixture may be agitated with stirrer 132, after which time the layers are allowed to separate. The first three way valve 120 is then opened and the bottom organic layer flows through outlet port 134, into a conductivity monitor 136 and then through tube 118 into chamber 114. The conductivity monitor is electronically coupled to the controller 126. A rise in conductivity indicates that the organic layer has passed through the conductivity monitor and the aqueous layer has begun to enter. The rise in conductivity is recognized by the controller 126 which sends a signal to the first three-way valve 120 actuating the three-way valve to divert the aqueous layer away from chamber 114.

Chamber 114 is equipped with three inlet ports 138, 140, and 142 and an outlet port 144. The organic layer enters chamber 114 through inlet port 138 and is pushed through the chamber 114 with a pressurized inert gas source, such as argon, which enters the chamber through inlet port 140. The chamber is then washed with solvent, *i.e.*,  $\text{CH}_2\text{Cl}_2$ ,

which enters the chamber through inlet port 142. The addition of solvent is controlled by the controller which selectively actuates the second valve 122. The organic effluent is collected through outlet port 144 by opening of the third valve 124 by the controller 126. The organic effluent contains the product of the reaction, which is the starting material 5 extended by one nucleotide and unreacted oligonucleotide starting material (failure sequence). The unreacted 5'- protected monomer is retained in the chamber 114. After elution of the organic solvent, the chamber 114 is washed with a buffered solution, added through inlet port 140, which elutes the unreacted 5'-protected monomer unit. Chamber 112 is then re-equilibrated with the organic solvent being used to elute the reaction mixture, *i.e.*, 10 CH<sub>2</sub>Cl<sub>2</sub>. The organic effluent is next passed over a reverse phase resin, to separate the product from the unreacted oligonucleotide starting material (failure sequence). (See Example 6).

Example 7 describes the solution phase synthesis of the 15 base oligonucleotide (5'-CTAAACGTAATGG-[3',3']-T-T-3') (SEQ ID NO:1) using polyethylene 15 glycol of 20,000 molecular weight as a 3' residue modification. This example demonstrates the efficiency of solution phase synthesis and the potential for preparing 3'-modified oligonucleotides in solution which can not be directly prepared using conventional solid phase synthesis. This example outlines the basic steps required for solution phase synthesis without the step wherein the oligonucleotide coupling product is captured on a resin as in a 20 typical PASS cycle. Thus, this example also demonstrates the impact on efficiency and product purity that product capture provides as envisioned in PASS. With such product capture at each monomer addition cycle, cumbersome precipitations from diethyl ether are no longer necessary as in conventional solid phase synthesis. Additionally, because failure sequences are removed at each monomer addition cycle, the anion exchange chromatogram 25 of the product obtained by PASS is expected to only show a single product peak, rather than the multiple peaks present in the chromatogram of Figure 6.

Example 8 (Schemes 7 and 8) describes the synthesis of various diene modified trityl alcohols including a 5'-di-(3,5-hexadienoxy)tritylthymidine phosphoramidite monomer (32) and a 5'-di-(2,4-hexadienoxy)tritylthymidine phosphoramidite monomer.

30 Example 9 (Scheme 9) demonstrates the use of dienes --4,4'-di-3,5-hexadienoxytrityl alcohol (30) and 4,4'-di-2,4-hexadienoxytrityl alcohol (36)-- for efficient

cycloaddition to maleimides (Reactions 1 and 2 respectively (Scheme 9)). Table 4 sets forth the reaction rates for these two reactions under various conditions. From the data set forth in Table 4, it is clear that modified trityl compound (30) reacts faster under the various reaction conditions. It is also clear that, as expected, both the increase in dienophile equivalents, as well as the addition of water to the reaction mixture increase the reaction rate. It is important to note reaction of greater than 50% of the diene substituents is sufficient for capture of all the trityl alcohol or nucleotide on a maleimide-modified solid phase support, since there are two dienes present on each trityl group. This reduces the time needed for the reaction to take place.

For rate comparison purposes the Diels-Alder reaction was carried out with 5'-O-(4,4'-di-3,5-hexadienoxytrityl)thymidine (5'-(DHDT)thymidine) (31) and with 5'-O-(4,4'-di-3,5-hexadienoxytrityl)thymidine 3'-phosphoramidite (32) under the same reaction conditions given for reaction #3 (Table 4). The results are set forth in Table 5. Again, within 1 hour more than 50% of the diene groups underwent cycloaddition. This suggests, that product capture, as envisioned in PASS, can occur within a reasonable time frame to allow rapid and efficient monomer addition cycles. It is widely known that the rate of Diels-Alder cycloadditions can be tailored by using suitably substituted dienes and dienophiles. Thus, the product capture reaction rate can be tailored by employing a suitable set of dienes and dienophiles.

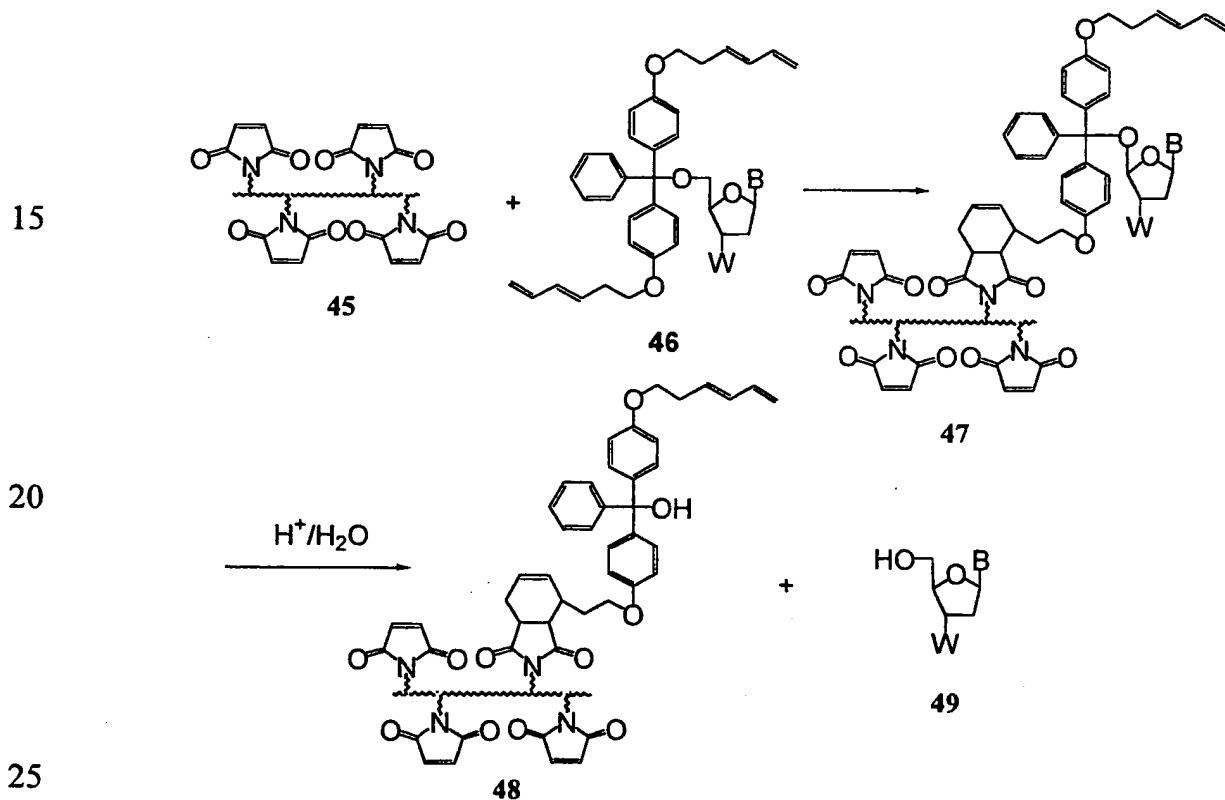
Example 10 describes the preparation of 3'-PEG derivatized oligonucleotides by PASS using the 4,4'-di-3,5-hexadienoxytrityl protecting group for capture of the oligonucleotide product on a substituted maleimide-polystyrene resin. This capture step removes the non-reacted starting oligonucleotide (failure sequence) from the reaction mixture. The latter can optionally be isolated and stored for blending into a subsequent production batch at the same point in the oligonucleotide assembly. A 3'-PEG terminal modification is useful *inter alia*, for enhancing the pharmacokinetic behavior of therapeutic oligonucleotides *in vivo*.

Example 11 describes a general reaction scheme for the preparation of non-PEG derivatized oligonucleotides by Diels-Alder product capture using a 5'-O-(4,4'-di-3,5-hexadienoxytrityl)-nucleoside (5'-O-DHDT-nucleoside) 46 as the diene and a maleimide substituted solid support 45 as the dienophile. (Scheme 11). As discussed above, the

capture of full length oligonucleotides on a resin or membrane is integral to automating the PASS process. The general design of the capture involves a trityl group or trityl analog being irreversibly bound to a solid support, such as a resin, membrane, or polymer 47. Once bound, the oligonucleotide 49 is released by separating it from the irreversibly bound trityl group 48. An example of this is the Diels-Alder capture of the 5'-O-DHDT-nucleoside described above. Resin-bound active Diels-Alder dienophiles covalently react with diene trityls and conventional methods of detritylation release the nucleoside from the solid support and bound trityl group. This capture can be employed to prepare non-PEG derivatized oligonucleotides by PASS as described in Example 11 (Scheme 12).

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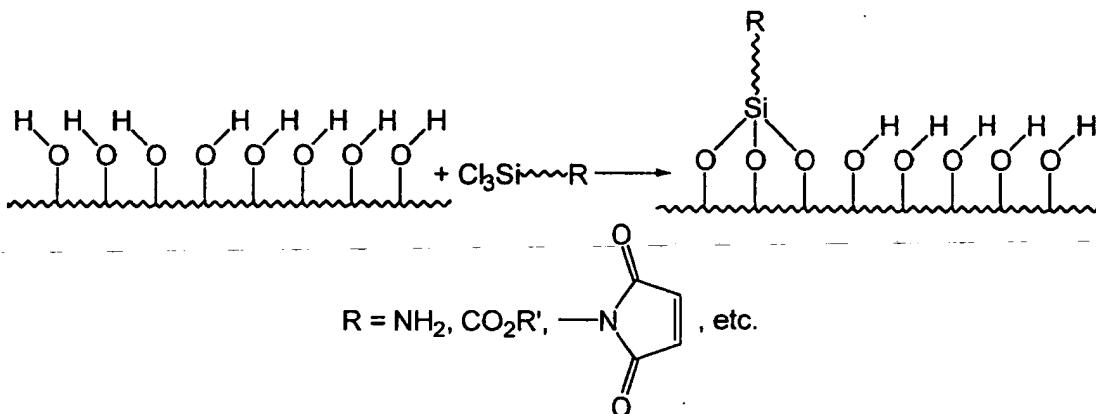
## SCHEME 11



A number of solid supports are envisioned to be suitable for capture and release using the Diels-Alder reaction. Preferred solid supports are inorganic oxides selected from the group consisting of silica, alumina, zeolites, controlled pore glass, that 30 have hydroxyl groups that are capable of being functionalized, as illustrated in Schemes 13 and 14. Inorganic oxides, such as silica, alumina, zeolites, controlled pore glass (CPG), etc.

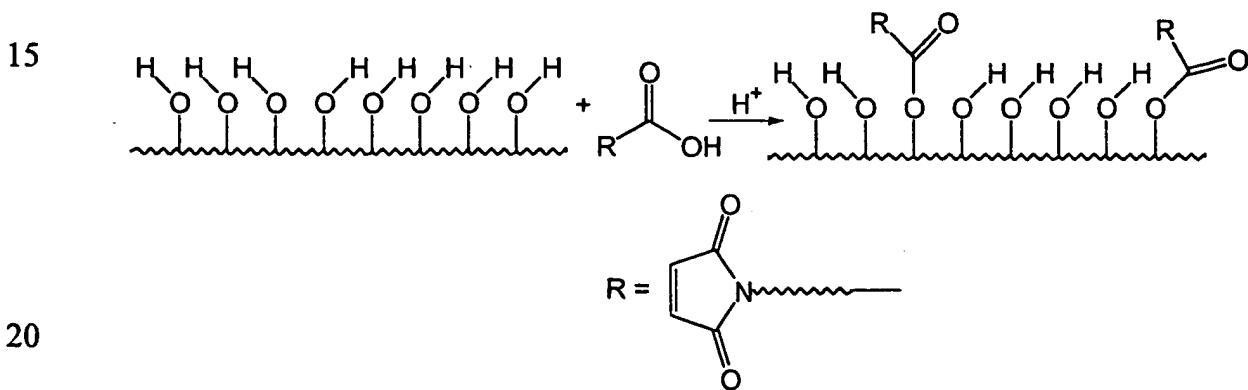
that have surface hydroxyl groups that can be readily functionalized. With the possible exception of CPG, these inorganic solid supports often have a much higher loading capacity than commercially available resins. Traditionally, these inorganic oxides have been functionalized by silylating the hydroxyls with a silylating agent that has a more versatile or reactive functional group. (Scheme 13).

### SCHEME 13



Other methods of covalently linking the reactive dienophile are also envisioned, for example, esterification between a molecule such as 6-maleimido-caproic acid and the surface hydroxyl group. (Scheme 14). Other covalent linkers between the surface and dienophile group may be used, if found to increase the surface loading and/or reactivity of the dienophile.

### SCHEME 14



Most preferably the solid support is an organic support such as polystyrene, or amino-functionalized polystyrene. These supports can be derivatized using standard

methods known to those in the art. As discussed below, Examples 18-21 (Schemes 23-26) illustrate two methods of preparing triazoline dione derivatized amino-functionalized resins.

Example 12 (Scheme 15) describes the preparation of a dimer using product capture by Diels-Alder cycloaddition. The rate of capture of the 3',3'-linked 5'-DHDTO-T-T dimer is dependent on the excess of resin bound maleimide groups. Product capture 5 proceeds quantitatively. The captured product is easily and quantitatively released from the resin with 3% dichloroacetic acid in dichloromethane. After neutralization and concentration, pure product is obtained.

Example 13 describes a method for assembly of oligonucleotides from 10 blocks by capturing one of the blocks on a resin using the cycloaddition of a 5'-O-(4,4'-di-3,5-hexadienoxytrityl) protected oligonucleotide to a dienophile derivatized resin.

Example 14 (Figure 8) illustrates schematically an automated 15 extraction/filtration system 200 and process designed for the automated preparation of an oligonucleotide bearing a 3'-terminal polyethylene glycol using covalent capture of the monomer addition product at every cycle, as described in Example 10. As discussed above, the PASS process, which consists of a controlled, sequential polymerization of nucleoside phosphoramidites, is ideally suited for automation. Each monomer addition consists of a sequence of chemical processing steps. This sequence remains the same for each monomer addition (cycle). The only variable from cycle to cycle is the nature of the monomer that is 20 added. A typical oligonucleotide consists of 2 to 12 different monomers, which are added typically more than once in a dedicated, programmable sequence.

As can be seen in Figure 8, the automated extraction/filtration system 200 has three centers: a reaction vessel 212, a filtration chamber 214 -- which contains the dienophile modified solid support 215 -- and an ultrafiltration membrane system 218.

Example 14 also lists various ultrafiltration membranes compatible with the 25 conditions required for the separation of a product oligonucleotide and excess monomer after release from the capture resin. Membranes are evaluated based on reagent/product adsorption, retention, and reactivity. The membranes set forth in Example 14 were found to be suitable, based on flux rates as affected by solvent, loss of product due to adsorption, and 30 finally by diffuse reflectance FTIR.

For purposes of illustration, the preparation of a 3'-terminal PEG oligonucleotide is described in Example 14, however, this automated method of synthesis can be done with or without a macromolecule attached to the oligonucleotide. In the latter case, the molecular weight cut-off membrane may be replaced by a liquid/liquid extraction 5 step, as depicted in Figure 5.

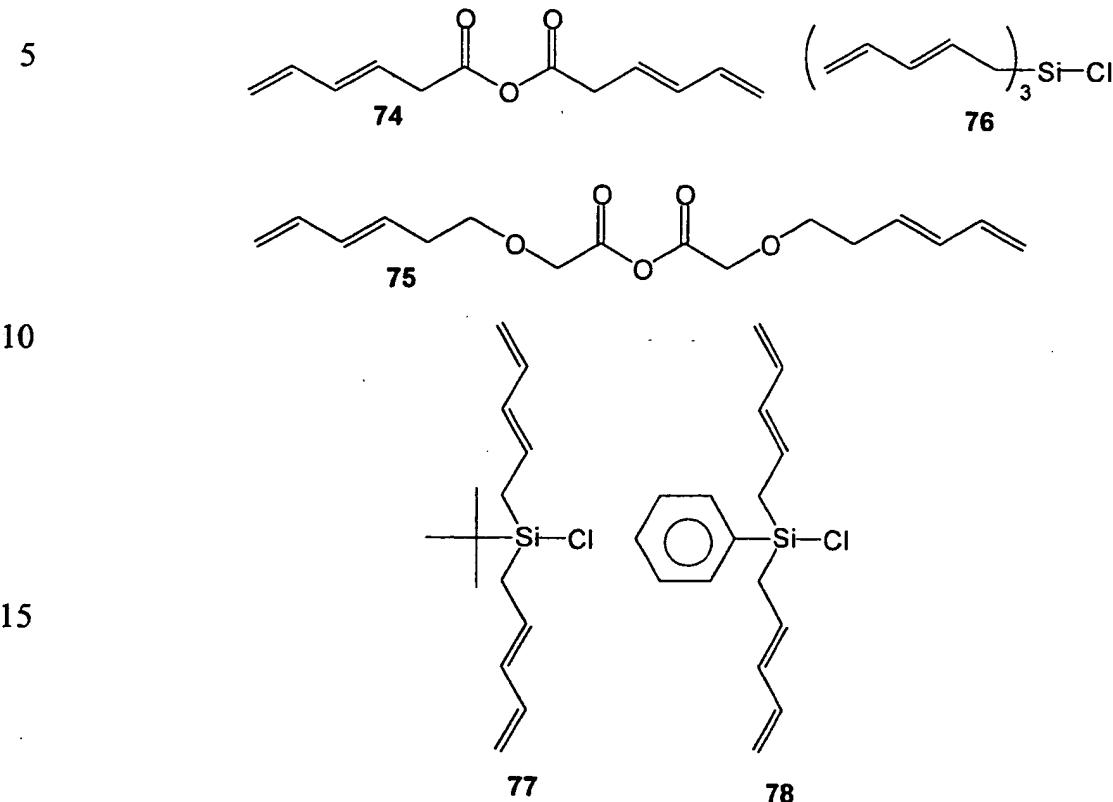
Example 15 describes the synthesis of maleimide derivatized trityl groups. As discussed above, an integral part of the PASS process is a method of removing n-1 sequences. One approach is oligonucleotide synthesis using monomers containing a maleimide-modified trityl group. These trityl groups are susceptible to reaction with diene-modified resins allowing separation of n-1 by simple washing of the resin followed by 10 detritylation to release the full-length oligonucleotide.

Example 16 describes the use of diene-modified capping reagents for the selective removal of failure sequences during solution phase synthesis and conventional solid phase synthesis. Typically, failure sequences are capped with acetic anhydride. The 15 capping reaction with acetic anhydride proceeds rapidly and near quantitatively. Thus, diene modified analogs of acetic anhydride, such as, 3,5-hexadienoic acid anhydride (74) and 3,5-hexadienoxyacetic anhydride (75) (Scheme 18) allows efficient capping of failure sequences and also enables removal of the capped failure sequence by cycloaddition to a dienophile derivatized resin or membrane at each cycle during solution phase synthesis as 20 described in Example 7. The 5'-acetyl capping groups introduced during conventional solid phase synthesis are removed during the ammonia cleavage and deprotection step. In order to utilize reagents 74 or 75 as capping reagents in solid phase synthesis and as subsequent handles for selective removal of the failure sequences, the oligonucleotide must be bound to the support via a linker, such as described in Example 12, which is selectively cleavable 25 under non-basic conditions. Alternatively, a capping reagent can be used which is not susceptible to removal under the typical basic deprotection conditions used at the end of conventional solid phase synthesis.

The hexadienoxy silyl chlorides (76, 77 and 78), allow selective removal of the failure sequences once the crude oligonucleotide is cleaved from the support with 30 ammonia. The silyl ether group is not removed under these conditions. Thus, the

hexadienoxy silyl capped failures can be removed from the desired product by reaction with a dienophile derivatized resin or membrane.

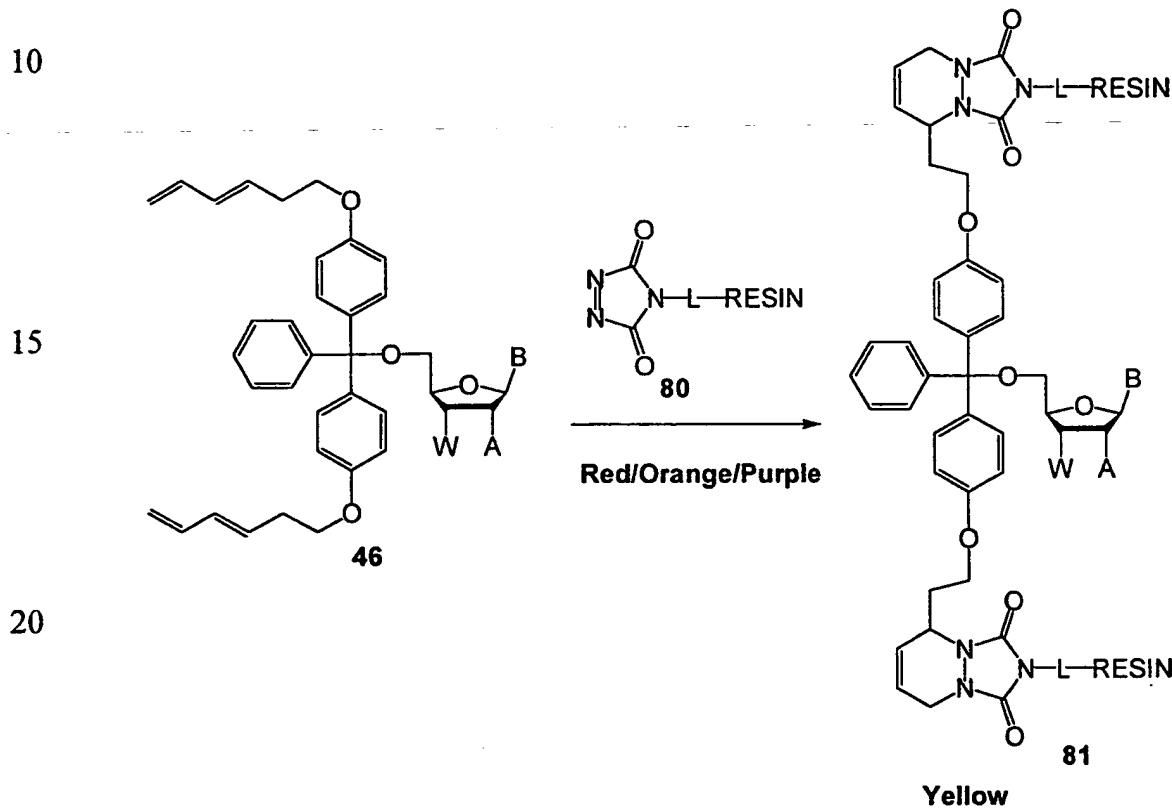
SCHEME 18



20 Scheme 19 illustrates generally a preferred method of this invention. As stated above, integral to the PASS process is the chemoselective isolation of the product after every monomer addition, preferably by covalent anchoring on a resin, polymer or membrane. In a preferred embodiment the method of covalent anchoring is via Diels-Alder reaction using a 1,2,4-triazoline-3,5-dione (TAD) modified resin as the dienophile resin component. The use of triazoline diones not only dramatically accelerates the anchoring reaction, but also allows visual monitoring of this crucial step. In this embodiment of the PASS process, a urazole is linked to a solid support. The immobilized urazole is oxidized to the triazoline dione with oxidizing agents such as NBS,  $N_2O_4$ , NCS or t-butyl hypochlorite. These TAD resins quickly react (within minutes at room temperature) with 25 hexadiene labeled trityl groups (DHDT) on the growing oligonucleotide. With reference to 30 Scheme 19 reaction of TAD resin **80** with a diene-trityl modified oligonucleotide product,

such as **46** produces the anchored product **81**. The TAD group, prior to anchoring by reaction with the diene-trityl modified product, displays a characteristic deep red/purple color. Upon anchoring by 2+4 cycloaddition reaction, the color shifts to pale yellow, such as in anchoring product **81**. Thus, complete product anchoring is simply achieved by 5 successive addition of the TAD-resin to the crude coupling product until the red color persists. The anchored product is released from the resin by traditional detritylation as described previously.

## SCHEME 19



In a preferred embodiment, a urazole-derivatized resin is oxidized to the corresponding 1,2,4-triazoline-3,5-dione resin (TAD-resin) prior to use with an excess of oxidant. The oxidant is removed from the resin by washing the resin with the appropriate solvents. When the oxidant is completely removed from the resin, the solvent in which the Diels-Alder capture reaction is to take place in is added to the resin to allow the resin to swell. The di-hexadienoxytrityl (DHDT)-derivatized oligonucleotide is then added to an excess of at least 2 equivalents of the resin per equivalent of DHDT. The reaction is monitored for the disappearance of the oligonucleotide by either TLC or HPLC.

Alternatively, the TAD-resin is added to the crude oligonucleotide product bearing a 5'-terminal DHDT group until the deep red color, characteristic of excess unreacted TAD-resin, persists. Upon determination of complete reaction by the aforementioned method(s), the resin is thoroughly washed with the appropriate solvents to remove any leachable material. The resin is then resuspended in the appropriate solvent and treated for 5 to 20 minutes with an acid sufficiently strong to effect detritylation and concomitant release of the 5'-deprotected oligomer. In the preferred embodiment of this invention, a solution of 3% dichloroacetic acid in dichloromethane is used. The resin is removed from the solution via filtration and the combined organic layers are repeatedly washed with a basic aqueous solution, preferably a  $\text{NaHCO}_3$  solution, followed by neutral aqueous solution, until the pH of the organic layer is between 6 and 7. The organic layer is then dried over  $\text{Na}_2\text{SO}_4$  and concentrated to yield the 5'-deprotected oligomer as a solid.

Example 17 (Scheme 22, Figure 10) describes the Diels-Alder reaction of the diene substituted trityl alcohol 5'-O-(4,4'-di-3,5-hexadienoxytrityl)thymidine (5'-DHDTO-dT) (31) with phenyltriazoline-dione (PTDA) (82). This example illustrates the accelerated reaction rates using triazoline diones and the potential for visual monitoring of the reaction.

Examples 18-21 (Schemes 23-26) illustrate two general methods for preparing triazoline dione derivatized resins. In the first method -- Examples 18 and 19 -- a urazole derivatized with benzoic acid is reacted with an amino-functionalized resin. In the second method, Example 20, a urazole is formed directly on the resin. Example 21 describes a variety of methods for oxidizing urazole functionalized solid supports. Example 22 (Scheme 27) describes the derivatization of a solid support with a urazole derivatized with caproic acid. Oxidation of this bound urazole with t-butyl hypochlorite produces the triazoline dione derivatized resin (99). Examples 19 and 22 illustrate the attachment of urazole to the solid support via an amide linkage. Urazole can also be attached to the solid support via an ester linkage.

An alternative approach to preparing TAD resins is to derivatize the solid support with a diene followed by reaction of the diene with an excess of a bis-TAD molecule. This approach, which is illustrated in Figure 11, generates a TAD resin without the need to oxidize the urazole to TAD on the solid support. This methodology is attractive due to the ease of resin functionalization, the availability of inexpensive starting materials

and the stability of the bis-TAD's allows for long term storage. The solid support can be selected from a resin, membrane or polymer. Examples include, but are not limited to silica, cellulose, polypropylene, polyvinyl alcohols, methacrylates, polystyrene and polyethylene glycol.

5 Example 23 (Schemes 28-30) illustrates the synthesis of a bis-TAD compound, the synthesis of a diene derivatized resin and the Diels-Alder reaction of the two compounds to generate a TAD resin without the need for on resin oxidation of the urazole to the TAD.

10 Other reactive dienophiles including, but not limited to thiocarbonyl compounds, such as thioketones, thioaldehydes, thioesters, thiocarbamates, thiocarbonates and thioamides,  $\beta$ -nitroacrylic acid and nitroso compounds can also be attached to a solid support and used in the capture release step. Thiocarbonyl compounds are highly reactive dienophiles, much more so than their carbonyl counterparts. Diels-Alder reactions of thiocarbonyl compounds proceed in good yield with butadienes at room temperature.

15 (Weinreb and Staib (1982) *Tetrahedron* 38:3087). Another reactive dienophile that can easily be covalently attached to a solid support is  $\beta$ -nitro acrylic acid. (Carruthers (1990) in Cycloaddition Reactions in Organic Synthesis, Pergamon Press, page 99). This dienophile also reacts with butadiene derivatives under mild conditions. Another potentially useful class of dienophiles are nitroso compounds. (Boger *et al.* (1985) *J. Org. Chem.* 50:19911).

20 These compounds can be generated *in situ* from benzohydroxamic acids by treatment with tetraalkyl periodate solutions. Once formed, the nitroso group quickly undergoes a Diels-Alder cycloaddition with butadiene.

25 Example 24 (Scheme 31) describes the use of a thiocarbonyl derivatized resin as the dienophile in the standard DHDT capture release cycle. This resin is easily prepared by standard synthetic organic methodologies and requires no oxidation step to produce the dienophile.

30 Example 25 (Scheme 32) describes the use of a nitroacrylate derivatized resin as the dienophile in the standard DHDT capture release cycle. Like the thiocarbonyl resin, the nitroacrylate derivatized solid support requires no preactivation. This resin is used in the standard DHDT capture release cycle.

Example 26 (Scheme 33) describes the use of a thioaldehyde resin generated *in situ* from a resin bound thiosulphinate. The thioaldehyde resin that is generated in the presence of the DHDT containing oligonucleotide is capable of undergoing a Diels-Alder reaction with the DHDT moiety thus capturing the desired product on the resin.

5 Example 27 (Scheme 34) illustrates the use of a nitrosoformate resin as the dienophile in the standard DHDT capture release cycle. The nitrosoformate resin is generated *in situ* from a hydroxycarbamic ester derivatized resin by the excess tetra alkyl periodate present in the oligo mixture from the oxidation of the phosphite triester to the phosphate triester. The nitrosoformate group is a very reactive dienophile and will quickly  
10 capture the DHDT moiety on the resin *via* a Diels-Alder cycloaddition reaction.

Figure 12 illustrates schematically one monomer addition cycle for Product Anchored Sequential Synthesis (PASS) of an oligonucleotide according to a preferred embodiment of this invention. Referring to this Figure, the starting material (a 3'-terminal oligonucleotide fragment) is coupled to the next 5'-protected monomer unit in the presence  
15 of an activator. Upon successful coupling, an organic soluble oxidizing agent, preferably tetrabutylammonium periodate, is added to the reaction mixture to oxidize the phosphite to a phosphate. Other oxidizing agents which are soluble in organic solvents, such as iodobenzene diacetate may also be used. Upon oxidation, the dienophile resin, preferably a triazoline-dione derivatized resin, is added. The anchoring reaction is allowed to proceed  
20 until complete anchoring of the coupling product on the resin is achieved. Subsequently, the resin is washed and exposed to a reagent that removes the resin-anchored, 5'-terminal protecting group, preferably the DHDT group, from the 5'-oxygen of the oligonucleotide fragment. This step, which is preferably carried out with dilute dichloroacetic acid, releases the product from the resin. The acid reagent is removed by passing the reaction mixture  
25 over a DOWEX resin. The product is isolated by precipitation. Upon dissolution in the reaction solvent, preferably a solvent mixture containing acetonitrile, the product is ready to undergo the next monomer addition.

Several alternatives to this cycle are equally as efficient. For example, the anchoring step may be performed prior to oxidation. In this instance, the resin-bound  
30 product is oxidized by exposure to an oxidizing agent that will convert a phosphite to a phosphahte species, such as pyridine buffered aqueous iodine. Additionally the DOWEX

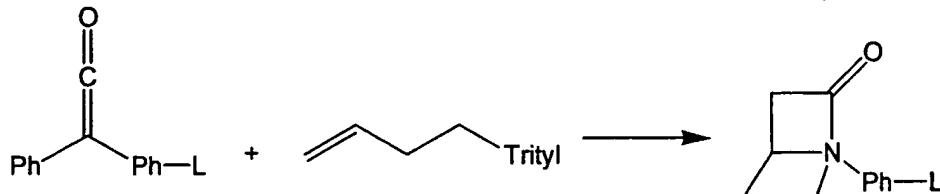
step for removal of excess dichloroacetic acid is not always necessary. Upon release from the resin, the product can be precipitated, and acid removal is achieved either by washing the precipitate, by trituration or by recrystallization of the precipitate.

Example 28 describes one monomer addition cycle of the PASS process  
5 wherein the product is covalently anchored to a 1,2,4-triazoline-3,5-dione (TAD) modified resin via Diels-Alder reaction.

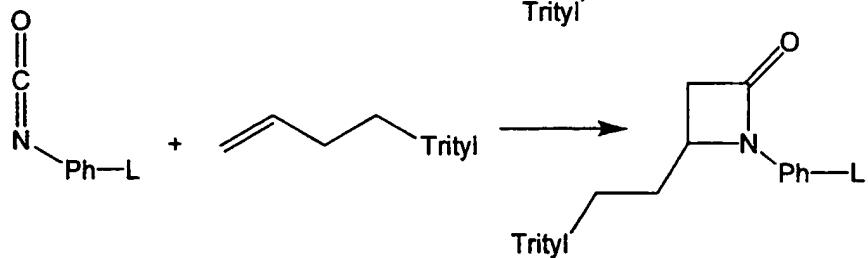
While the Diels-Alder reaction is a preferred method for anchoring the product of the reaction to a solid support, other methods are also contemplated as being within the scope of this invention. For example, a 2+3 dipolar cycloaddition reaction may  
10 be applied to the anchoring step. In this embodiment an alkene group, either part of the D group on the oligonucleotide product or on the resin, is reacted with a 1,3-dipolar substituent to form the cyclic five-membered addition product. A 2+2 cycloaddition reaction may also be applied to the anchoring step. (Scheme 20).

SCHEME 20

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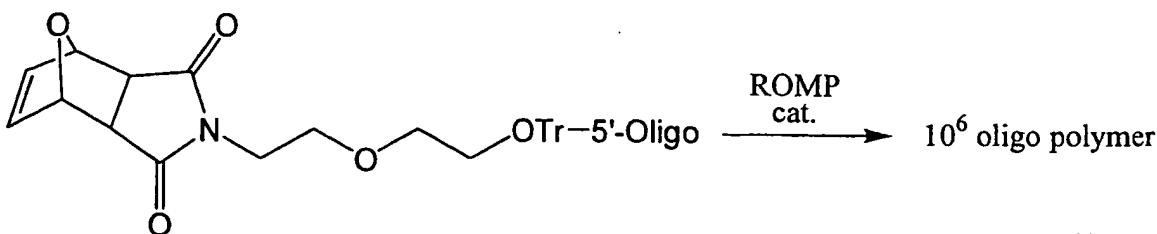
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25 In this embodiment a solid support derivatized with a ketene (isocyanate or other reactive cummulene) is prepared and treated with an olefin modified 5'-trityl oligonucleotide. The recently discovered ring-opening metathesis polymerization (ROMP) of 7-oxanorbornenes also has potential application in the capture/release step of the PASS process. (Scheme 21).

30

## SCHEME 21



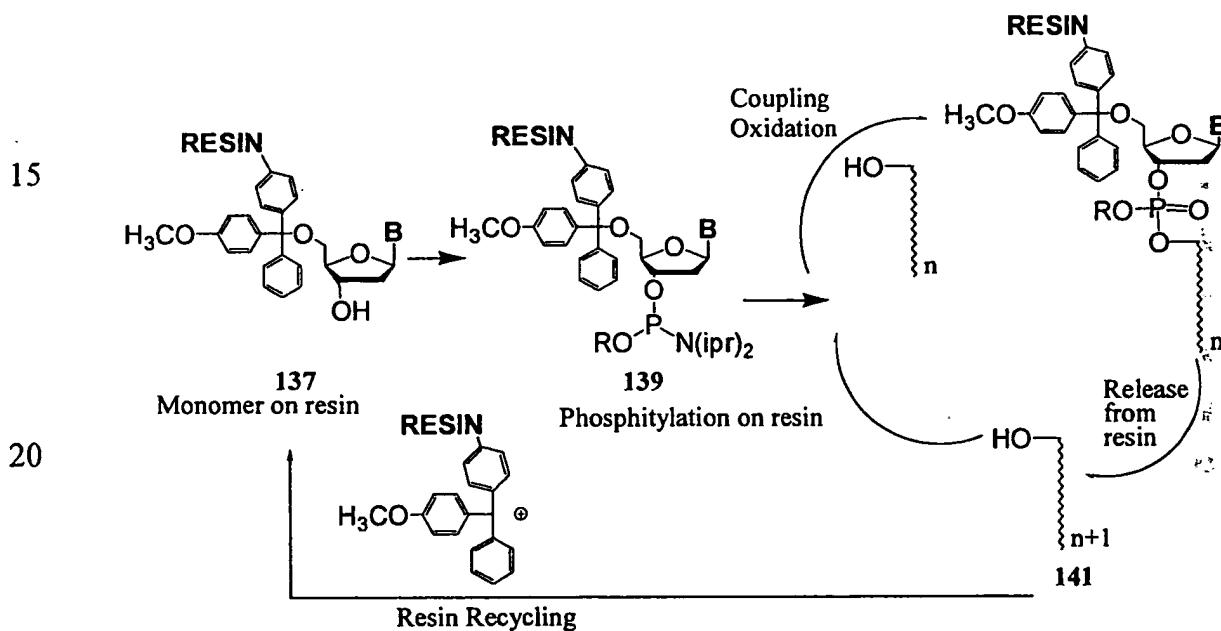
In this example a 7-oxanorbornene derivatized trityl is prepared and coupled to the 5' alcohol of a nucleoside. The phosphoramidite (or H-phosphonate) is prepared and is then used in a standard coupling cycle. After coupling (and oxidation for 5 phosphoramidites) the ROMP polymerization is carried out and the resulting polymeric solution filtered and washed. The polymer is then treated with the  $\text{CH}_2\text{Cl}_2/\text{DCA}$  mixture to liberate the full length oligonucleotide.

Example 29 (Scheme 35) describes a general synthesis of diene-ols using hydroboration.

10 Scheme 36 illustrates oligonucleotide synthesis using product anchoring by coupling to a resin bound monomer. This preferred embodiment retains the desired aspects of the PASS process without the need for producing diene trityl nucleoside phosphoramidites and without the need of preparing the triazoline dione resin. N-protected 5' and 3' hydroxyl nucleosides are known to react selectively at the 5' hydroxyl group with 15 trityl chloride. (Beaucage and Radhakrishnan (1992) *Tetrahedron* **48**:2223). Taking advantage of this selectivity one can use a commercially available trityl chloride resin (available from Calbiochem-Novabiochem) and attach an N-protected nucleoside at the 5' hydroxyl group producing a 5' tritylated nucleoside directly attached to a solid support (137). This resin bound nucleoside can be phosphitylated at the 3' end by treatment with 20 excess phosphitylating reagent. Since the nucleoside is attached to a solid support, the excess reagents can be easily removed by a wash step. The resulting phosphoramidite resin (139) can then be treated DCI and with a 5' hydroxyl N-protected, 3' protected nucleoside (or 5' hydroxyl oligonucleotide with all other sites protected) to produce a resin bound phosphotriester dimer (or in the case of the 5' hydroxyl oligonucleotide, the  $n+1$  mer of the 25 oligonucleotide). Any of the unreacted 5' hydroxyl (monomer or oligo) can be recovered by washing the resin. The resulting phosphite triester is oxidized to the phosphate triester by

washing the resin with the appropriate oxidizing solution (either iodine or tetra alkyl periodate or for forming phosphorothioates the standard Beaucage reagent can be used). The desired product 141 can then be cleaved from the resin by running the standard deblock cycle. The trityl group stays bound to the resin (as in the Diels-Alder capture release 5 example) and the product is washed off in the methylene chloride/dichloroacetic acid solution. Extraction of the acidic wash with aqueous phosphate buffer (pH=7.0) removes the dichloroacetic acid as well as any of the unreacted resin bound monomer (either as the nucleoside or the hydrolyzed phosphoramidite). The methylene chloride layer can then be evaporated to give the pure dimer (or  $n+1$  mer oligonucleotide with the free 5' hydroxyl 10 group). This cycle can be repeated until the desired oligo length is achieved.

SCHEME 36



25 This embodiment is not limited to the particular trityl resin shown or to the particular coupling chemistry described above. Rather, in a more generic sense, the present invention describes the preparation of a linear, sequential polymer by anchoring of the incoming monomer to a resin, soluble polymer, membrane, or immiscible liquid phase. The attachment of the incoming monomer is achieved through a removable protecting 30 group, preferentially the 5'-protecting group in nucleoside monomers for oligonucleotide synthesis. In peptide synthesis, the monomer is preferentially anchored through the N-

terminal protecting group. The growing polymer chain is attached to the anchored monomer by standard coupling chemistry, such as phosphoramidite, H-phosphonate or phosphate triester coupling in case of oligonucleotide synthesis. Upon successful coupling all reagents and any remaining uncoupled polymer starting material are removed by simply washing the 5 anchored product. This step is important, since it effects the purification of the growing polymer after every monomer addition without the need for complex purification techniques. The extended polymer chain is then released from the resin, soluble polymer, membrane or immiscible liquid phase by a reagent that cleaves the protecting group from the extended polymer chain.

10 Example 30 (Schemes 37-39) illustrates the use of a trityl resin in the PASS process. In this example, the trityl chloride resin is derivatized with a thymidine nucleoside (through the 5'-hydroxyl), the resin bound nucleoside is phosphitylated at the 3'- hydroxyl and then treated with a slight excess of 5'-diphenyl t-butyl silyl thymidine nucleoside (3' OH) in the presence of DCI activator. After the coupling and oxidation steps are complete 15 the resin is washed to remove all unreacted material. The product (T-T dimer) is then removed from the resin by the usual detritylation method. Example 30 also illustrates the preparation of a trimer using this method.

20 Example 31 illustrates the application of the PASS process to a high resolution, single-step method of purification. In this example, a 5'-DHDT derivatized nucleoside is protected at the 3'-position with a tertbutyldiphenylsilyl group followed by 25 purification using the PASS process. As set forth in Table 10 capture was 100% and the purity of the final product was 91%. This example demonstrates that column-based purifications can be replaced using the capture-release techniques employed in the PASS process.

25 The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention.

**Example 1. Preparation of N-4-benzoyl-3'-(5'-tert-butyldimethylsilyl-3'-(2-cyanophosphoryl)thymidyl)-2'-fluorocytidine (16) (Scheme 4)**

30 5'-tert-butyldimethylsilylthymidine 12 (5'-TBDMS-thymidine) (0.15 g, 0.42 mmol) was dissolved in dry acetonitrile (10 mL) under an argon atmosphere. Cytidine

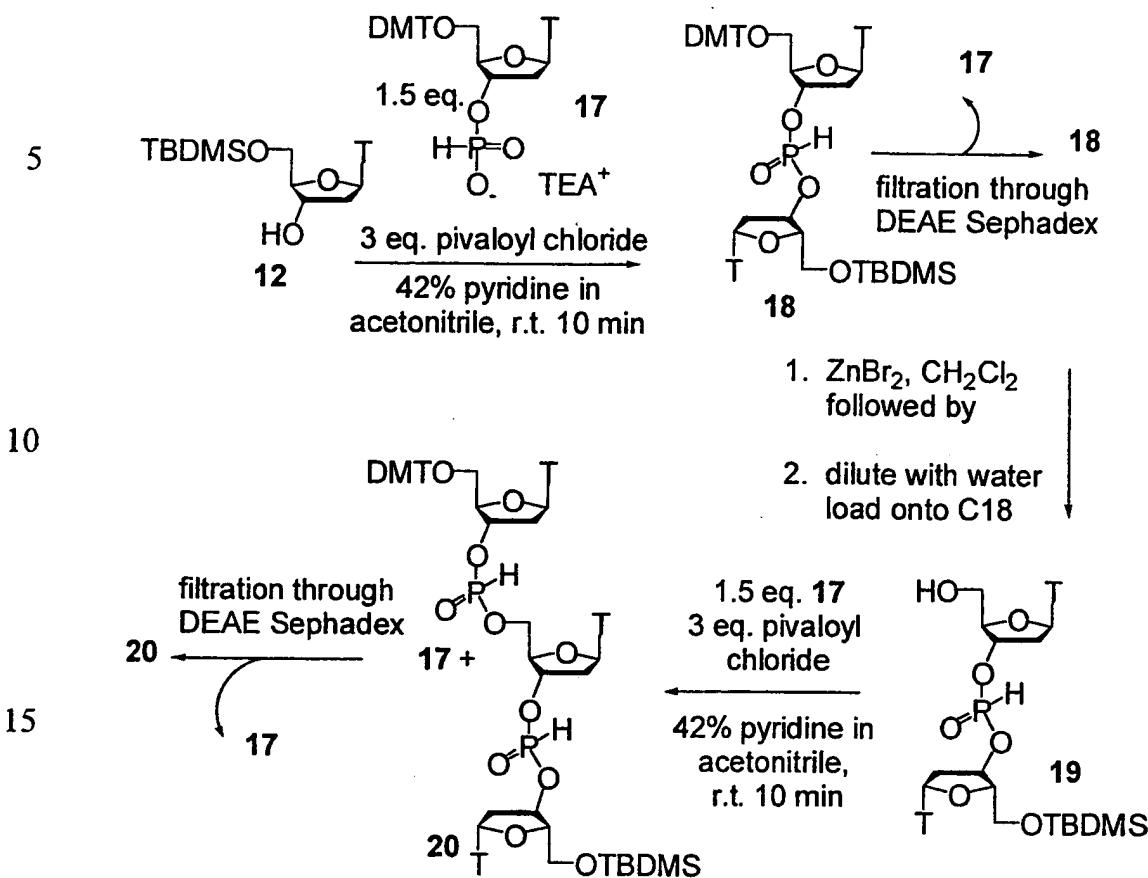
amidite **13** was added (0.43 g, 0.50 mmol) followed by tetrazole (6.5 mL, 0.45 M in acetonitrile). After 15 minutes reverse phase HPLC analysis (C18, 4.6x100 mm, Buffer A: 100 mM triethylammonium acetate pH 7.5, Buffer B: acetonitrile, 0 to 80% B over 2.5 minutes) of the reaction mixture showed the presence of dimer (2.4 minutes) as well as 5 unreacted thymidine **12** (1.4 minutes) and hydrolyzed amidite monomer (2.1 minutes) (Figure 1). The reaction mixture was oxidized *in situ* (10 mL, 0.2 M iodine in water/pyridine). HPLC analysis after oxidation reveals the presence of pyridine (0.9 minutes), unreacted thymidine **12** (1.4 minutes), oxidized amidite monomer **15** (1.8 minutes), and oxidized dimer **14** (2.3 minutes) (Figure 2).

10 After oxidation the reaction mixture was passed with acetonitrile through a bed of DEAE Sephadex® pre-equilibrated with acetonitrile. HPLC analysis of the filtrate indicates retention of the oxidized amidite monomer **15** as shown in Figure 3. The filtrate was concentrated under reduced pressure and the solid was re-dissolved in 60% acetonitrile/water and loaded onto a C18 chamber pre-equilibrated with 70% 15 water/acetonitrile. The chamber was washed with 70% water/acetonitrile followed by 50% water/acetonitrile to fully elute the unreacted thymidine **12**. The chamber was then washed with water and treated with 80% acetic acid/water to effect detritylation. Following detritylation the chamber was washed with 50% acetonitrile/water to elute the final product **16** (m/e 922, product **16** plus triethylamine). HPLC analysis shows elution of the 20 detritylated species **16** at 1.7 minutes (Figure 4). ESMS (Electrospray Mass Spectrometry) of **16**: Calcd 820.27 (M+); Found 922.2 (M+H+TEA).  $^{31}\text{P}$  NMR (121 MHz,  $\text{CDCl}_3$ ,  $\text{H}_3\text{PO}_4$  external standard)  $\delta$  -0.73, -1.93. The trityl species was retained on the chamber.

Example 2. Preparation of a H-phosphonate thymidine trimer (T-T-[3',3']-T) (20)

25 Assembly of a H-phosphonate thymidine trimer bearing a 3',3'-internucleotidic linkage at the 3'-terminus was synthesized as outlined in Scheme 5.

## SCHEME 5

Coupling of 12 to 5'-dimethoxytritylthymidine 3'-H-phosphonate 17. To a

20 solution of 17 (0.75 g, 1.05 mmol) in 1:1 acetonitrile:pyridine (42 mL) under argon was added 12 (0.25 g, 0.7 mmol), followed by a solution of pivaloyl chloride (0.26 mL, 2.1 mmol) in 95:5 acetonitrile:pyridine (8.4 mL). The reaction was stirred for 10 minutes, at which time reverse phase HPLC analysis showed complete conversion of 12 to dimer 18. The mixture was then concentrated *in vacuo*, dissolved in CH<sub>2</sub>Cl<sub>2</sub>, and extracted with 0.05 M triethylammonium bicarbonate. The methylene chloride layer was applied to a plug of DEAE Sephadex® on a Buechner funnel. Reverse phase HPLC analysis of the filtrate showed complete removal of the unreacted monomer 17. Dimer 18 was isolated in quantitative yield, by evaporation of the filtrate and its structure was confirmed by NMR and ESMS analysis. Unreacted monomer 17 was recovered from the DEAE Sephadex® plug by washing with 1 M triethylammonium bicarbonate. ESMS of 18: Calcd 946.4 (M+); Found 946.3. <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>CN) δ 9.21 (s, 2H), 7.45-7.24 (m, 11H), 6.94 (d,

1H,  $J=717.2$  Hz), 6.89-6.85 (m, 4H), 6.31-6.19 (m, 2H), 5.22-5.19 (m, 1H), 5.05-5.00 (m, 1H), 4.22-4.19 (m, 1H), 4.11-4.10 (m, 1H), 3.81-3.80 (m, 2H), 3.75 (s, 6H), 3.36-3.35 (m, 2H), 2.52-2.17 (m, 4H), 1.83 (s, 3H), 1.47 (s, 3H), 0.91 (s, 9H), 0.10 (s, 6H).  $^{31}\text{P}$  NMR (121 MHz,  $\text{CD}_3\text{CN}$ )  $\delta$  14.03 (d), 13.88 (d).

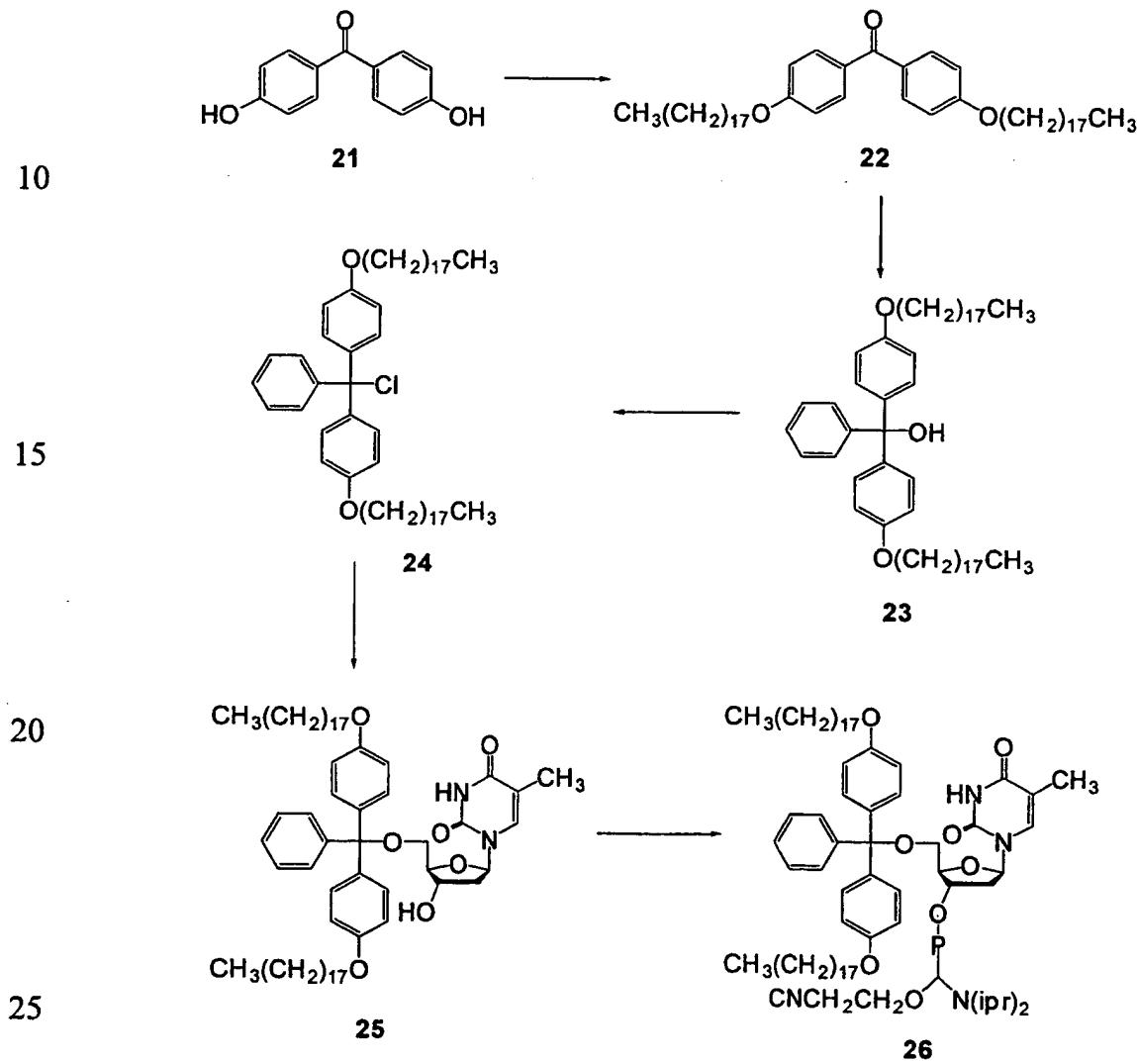
5 Detritylation of dimer 18. Dimer 18 (0.85 g, 0.9 mmol) was dissolved in methylene chloride saturated with  $\text{ZnBr}_2$  (10 mL, approximately 0.1 M  $\text{ZnBr}_2$ ). After 15 minutes reverse phase HPLC analysis showed complete detritylation. The reaction was quenched with an equal volume of 1 M ammonium acetate. The organic layer was concentrated, the residue dissolved in 1:1 acetonitrile:water and passed over a C18 plug on a 10 Buechner funnel. Evaporation of the filtrate gave 0.29 g (50 % yield) of pure dimer 19. ESMS of 19: Calcd 644.2 (M+); Found 645.3.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  10.0, 9.85, 9.55, 9.45 (4s, 2H), 7.59-7.45 (m, 2H), 7.01 (d, 1H,  $J=712.3$  Hz), 6.39-6.19 (m, 2H), 5.35-5.23 (m, 1H), 5.14-5.03 (m, 1H), 4.31-4.22 (m, 2H), 3.88-3.79 (m, 4H), 2.67-2.48 (m, 3H), 2.21-2.12 (m, 1H), 1.89-1.88 (2bs, 6H), 0.90 (s, 9H), 0.11 (s, 6H).  $^{31}\text{P}$  NMR (121 15 MHz,  $\text{CDCl}_3$ )  $\delta$  8.45 (d), 8.30 (d).

15 Preparation of trimer 20. To a solution of dimer 19 (0.25 g, 0.39 mmol) in 1:1 pyridine acetonitrile (23 mL) was added 17 (0.41 g, 0.58 mmol), followed by a solution of pivaloyl chloride (0.14 mL, 1.16 mmol) in 95:5 acetonitrile:pyridine (4.5 mL). The reaction was stirred under an argon atmosphere for 10 minutes, at which point HPLC 20 analysis indicated complete conversion of dimer 19 to trimer 20. The mixture was evaporated to dryness, dissolved in  $\text{CH}_2\text{Cl}_2$ , washed with 0.05 M triethylammonium bicarbonate, and the organic layer was applied to a DEAE Sephadex® plug on a Buechner funnel. The filtrate was evaporated to give 20 in quantitative yield. ESMS of 20: Calcd 1234.4 (M+); Found 933.5 (M+H+with loss of DMT).  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{CN}$ )  $\delta$  9.34-9.27 (m, 2H), 8.58-8.56 (m, 2H), 8.18-8.11 (m, 1H), 7.76-7.70 (m, 1H), 7.43-7.41 (m, 4H), 7.35-7.23 (m, 13H), 6.88-6.84 (m, 4H), 6.26-6.15 (m, 3H), 5.78-5.71 (m, 1H), 5.22-5.20 (m, 1H), 5.11-5.05 (m, 2H), 4.29-4.26 (m, 2H), 4.24-4.19 (m, 2H), 3.85-3.84 (m, 2H), 3.76 (s, 6H), 3.74-3.72 (m, 1H), 3.38-3.28 (m, 2H), 2.52-2.20 (m, 6H), 1.82 (s, 3H), 1.78 (s, 3H), 1.47-1.44 (m, 3H), 0.90 (s, 9H), 0.11 (s, 6H).  $^{31}\text{P}$  NMR (121 MHz,  $\text{CD}_3\text{CN}$ )  $\delta$  30 15.86 (s), 15.08 (s), 14.36 (s).

Example 3. Preparation of 5'-O-(4,4'-dioctadecyltriphenylmethyl)thymidine-3'-O-(N,N-diisopropyl-2-cyanoethylphosphoramidite (26)

Assembly of a phosphoramidite monomer containing 4,4'-dioctadecyltriphenylmethanol (DOT) as the 5'-protecting group (D-E) is illustrated in 5 Scheme 6.

SCHEME 6



4,4'-Dioctadecyloxy-benzophenone (22). Sodium metal (0.46 g, 20 mmol) was dissolved in ethanol (50 mL) and 4,4'-dihydroxybenzophenone (21) (1.0 g, 4.67 mmol) was added followed by 1-bromooctadecane (7.8 g, 23.4 mmol) and a catalytic amount of 30 sodium iodide (approximately 30 mg) and the reaction mixture was refluxed for 48 hours. The resulting suspension was cooled and filtered. The solid was washed with

dichloromethane followed by hexane and the white solid was dried to afford compound 22 (2.85 g, 84.8% yield).  $^1\text{H}$  NMR (300 MHz, pyridine-d<sub>5</sub>)  $\delta$  7.95 (d, J=8.7 Hz, 4H, aryl), 7.09 (d, J=8.7 Hz, 4H, aryl), 4.05 (t, J=6.6 Hz, 4H, 2xOCH<sub>2</sub>), 1.80 (tt, J= 6.6 and 7.5 Hz, 4H), 1.48 (m, 4H), 1.33 (brS, 60H), 0.87 (t, J=6.6 Hz, 6H, 2xCH<sub>3</sub>).

5 4,4'-Dioctadecyltriphenylmethanol (23). To a suspension of benzophenone 22 (0.3 g, 0.42 mmol) in anhydrous THF (4 mL) was added phenylmagnesium bromide (0.55 mL, 1.0 M solution in THF, 0.55 mmol) and the reaction was refluxed for 3 hours. An additional amount of phenylmagnesium bromide (0.2 mL) was added and the heating was continued for 0.5 hours at which time all of the starting material had dissolved. The 10 reaction was then cooled and 0.5 M HCl was added. The suspension was filtered and the solid washed with water (3x), hexane (2x) and dichloromethane (2x). The organic washes were pooled, dried (MgSO<sub>4</sub>) and evaporated to afford 23 (0.21 g, 63.6% yield) as a white solid.  $^1\text{H}$  NMR (300 MHz, pyridine-d<sub>5</sub>)  $\delta$  8.13 (brS, 1H, aryl), 7.81 (d, J=7.1 Hz, 2H), 7.7 (d, J=8.8 Hz, 4H), 7.42 (t, J=7.7 Hz, 2H), 7.34 (t, J=7.1 Hz, 1H), 7.07 (d, J=8.9 Hz, 4H), 15 3.98 (t, J=6.4 Hz, 4H), 1.77 (tt, J=7.9 and 6.5 Hz, 4H), 1.45 (m, 4H), 1.3 (brS, 60H), 0.87 (t, J=6.9 Hz, 6H).

15 5'-O-(4,4'-Dioctadecyltriphenylmethyl)thymidine (25). Compound 23 (2.1 g, 2.63 mmol) was coevaporated twice with toluene then dissolved in toluene (30 mL). Acetyl chloride (11 mL, 154.7 mmol) was added and the reaction was refluxed for 3 hours and then 20 evaporated. The residue was coevaporated twice with toluene to afford crude 24. To 24 was added pyridine (30 mL), DMAP (25 mg) and thymidine (0.45 g, 1.86 mmol) and the reaction was stirred at room temperature overnight. The solvent was evaporated under reduced pressure and the residue was taken up in dichloromethane and washed with 5% sodium bicarbonate. The organic phase was dried (MgSO<sub>4</sub>) and evaporated and the residue 25 was purified on silica gel (ethyl acetate/2% triethylamine) to afford after evaporation of the appropriate fractions compound 25 (DOT thymidine) (1.6 g, 84% yield) as pale yellow solid.  $^1\text{H}$  NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  8.44 (brS, 1H, NH), 7.60 (s, 1H, H-6), 7.41-7.20 and 6.82 (m, 13H, DOT), 6.42 (t, J=6.1 Hz, 1H, H-1'), 4.57 (m, 1H, H-3'), 4.05 (m, 1H, H-4'), 3.92 (t, J=6.5 Hz, 4H, 2xOCH<sub>3</sub>), 3.47 and 3.37 (ABX, 2H, H-5'), 2.38 (m, 2H, H-2'), 2.22 (m, 1H, 3'-OH), 1.75 (m, 4H, DOT), 1.46 (m, 7H, 5-CH<sub>3</sub>, DOT), 1.25 (brS, 60H, DOT), 30 0.87 (t, 6H, 2xCH<sub>3</sub>).

5'-O-(4,4'-Dioctadecyltriphenylmethyl)thymidine-3'-O-(N,N-diisopropyl-2-cyanoethylphosphoramidite (26). DOT thymidine 25 was dissolved in dichloromethane (5 mL) and diisopropylethylamine (0.3 mL, 1.75 mmol) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.15 mL, 0.63 mmol) was added with ice bath

5 cooling. The ice bath was removed and the reaction was stirred at room temperature for 4 hours at which point additional 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.1 mL) was added and the reaction was stirred for 16 hours at room temperature. The reaction solution was diluted with dichloromethane and washed with 5% sodium bicarbonate, the organic phase was dried ( $\text{MgSO}_4$ ) and evaporated. The residue was purified on silica

10 eluting first with hexanes, followed by 20% ethyl acetate/hexanes all containing 2% triethylamine to afford 26 as resolved diastereomers (fast 0.1 g, slow 0.18 g, 46.7 % yield). **26a** (fast diastereomer)  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.15 (br, 1H, NH), 7.91 (s, 1H, H-6), 7.63, 7.38-7.21, 6.82 (m, 13H, aryl), 6.39 (t,  $J=7.4$  Hz, H-1'), 4.67 (m, 1H, H-3'), 4.18 (m, 1H, H-4'), 3.92 (t,  $J=6.5$  Hz, 2xOCH<sub>3</sub>), 3.63 (m, 4H), 3.51 and 3.33 (ABX, 2H, H-5'), 2.42 and 2.26 (m, 4H, H-2', CH<sub>2</sub>CN), 1.73 (m, 4H), 1.41 (m, 4H), 1.25 (s, 60H), 1.16 (dd,  $J=2.7$ , 6.9 Hz, 12H), 0.87 (t,  $J=6.9$  Hz, 6H).  $^{31}\text{P}$  NMR (121 MHz,  $\text{CDCl}_3$ )  $\delta$  150.64. **26b** (slower diastereomer)  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  8.25 (br, 1H, NH), 7.91 (s, 1H, H-5), 7.41 and 7.31-7.20 and 6.81 (m, 13H, aryl), 6.42 (dd,  $J=8$  Hz, H-1'), 4.67 (m, 1H, H-3'), 4.14 (m, 1H, H-4'), 3.92 (t,  $J=6.5$  Hz, 4H, 2xOCH<sub>2</sub>), 3.82 and 3.76 (m, 2H), 3.54 (m, 2H), 3.47 and 3.31 (ABX, 2H, H-5'), 2.62 (t,  $J=6.3$  Hz, 2H, CH<sub>2</sub>CN), 2.54 and 2.34 (m, 2H, H-2'), 1.76 (m, 4H), 1.25 (m, 60H), 1.16 and 1.05 (d,  $J=6.9$  Hz, 12H, isopropyl CH<sub>3</sub>), 0.87 (t,  $J=6.6$  Hz, 6H).  $^{31}\text{P}$  NMR (121 MHz,  $\text{CDCl}_3$ )  $\delta$  150.23.

Example 4. Resolution of alkyl substituted trityl groups on reverse phase resin

25 The alcohols of 4,4'-dioctadecyltriphenylmethanol (DOT), 4-decyloxy-4'-methoxytritanol, and dimethoxytritanol (DMT) were spotted onto a C18 reverse phase TLC plate and the plate was developed in three different solvents (Table 1). As can be seen in Table 1, there is a strong interaction of the DOT group with the C18 resin in organic solvents, such as methanol ( $R_f=0$ ) and acetonitrile ( $R_f=0$ ). This interaction enables the one-step separation of the coupled product from starting material based upon the affinity or interaction of the trityl protecting group for C18 reverse phase resin.

Example 5. Preparation of 5'-HO-T-T-A-C-T-[3',3']-T-3' by PASS using hydrophobic affinity for product capture

Preparation of 5'-HO-T-[3',3']-T. 5'-TBDPS-thymidine **12** (0.99 g, 2.07 mmol) was co-evaporated with dry methylene chloride and dissolved in 10 mL of dry 5 methylene chloride. Thymidine amidite (2.0 g, 2.69 mmol) was added followed by tetrazole 0.5 M in acetonitrile (21 mL, 10.5 mmol) and the reaction was stirred under argon. After 90 minutes, a solution of iodine/water/pyridine (0.2 M) was added until the dark brown color persisted, followed by 5% NaHSO<sub>3</sub> until the color returned to yellow. The concentrated reaction was partitioned (CH<sub>2</sub>Cl<sub>2</sub>/water) and the organic layer was dried with MgSO<sub>4</sub> and 10 evaporated to dryness. The solid residue was dissolved in methanol/minimal methylene chloride and pipetted onto a 75 g bed of DEAE Sephadex® equilibrated with water then methanol. The DEAE Sephadex® was washed with 300 mL methanol and the combined methanol washes were concentrated to afford 2.42 g of a white foam.

**Detritylation:** The white foam was dissolved in 50 mL of 3% DCA and stirred at room 15 temperature for 35 minutes, and then poured over 80 mL of silica gel equilibrated with methylene chloride. The gel was washed with 150 mL of 3% DCA, followed by solutions from 100% methylene chloride through 6% methanol in methylene chloride. Appropriate fractions were combined and concentrated to give 1.58 g of detritylated dimer (**5'-HO-T-[3',3']-T**) in 90% yield for the two step process.

Preparation of the 5'-HO-C-T-[3',3']-T. The 5'-HO-T-[3',3']-T dimer (1.47 g, 20 1.76 mmol) was dried under high vacuum overnight, and then co-evaporated with dry CH<sub>2</sub>Cl<sub>2</sub> and dissolved in 8.5 mL of dry CH<sub>2</sub>Cl<sub>2</sub>. Cytidine amidite (1.90 g, 2.28 mmol) was added followed by tetrazole (0.5 M) in acetonitrile (17.6 mL, 8.78 mmol) and the reaction was stirred under argon. After 50 minutes, a 0.5 M iodine solution was added, followed by 25 5% NaHSO<sub>3</sub>, changing the color from brown to yellow as described above. The concentrated reaction was partitioned (CH<sub>2</sub>Cl<sub>2</sub>/water) and the organic layer was dried (MgSO<sub>4</sub>) and evaporated to dryness. The solid residue was dissolved in methanol/minimal methylene chloride and pipetted onto a 75 g bed of DEAE Sephadex® pre-equilibrated with water and then methanol. The DEAE Sephadex® was washed slowly with methylene 30 chloride and methanol and the combined washes were concentrated to afford 2.53 g of a yellow foam.

**Detritylation:** The foam was stirred in 50 mL of 3% DCA at room temperature. After 2 hours, the reaction mixture was pipetted onto an 80 mL bed of silica gel pre-equilibrated with methylene chloride. The mixture was eluted with 3% DCA, followed by solutions from 100%  $\text{CH}_2\text{Cl}_2$  through 6% methanol in  $\text{CH}_2\text{Cl}_2$ . The appropriate fractions were 5 combined and concentrated to give 1.43 g of the detritylated trimer (5'-HO-C-T-[3',3']-T), 64% yield for the two step process.

**Preparation of 5'-HO-A-C-T-[3',3']-T.** The detritylated trimer 5'-HO-C-T-[3',3']-T (1.43 g, 1.1 mmol) was dried under high vacuum overnight, coevaporated with dry methylene chloride and dissolved in 6 mL of dry methylene chloride. Adenine amidite (1.24 10 g, 1.45 mmol) was added, followed by 0.5 M tetrazole in acetonitrile (11 mL, 5.57 mmol) and the reaction was stirred under argon. After approximately 60 minutes, a 0.5 M iodine solution was added until the dark color persisted. The mixture was then stirred for 1 hour and concentrated. The gum was partitioned ( $\text{CH}_2\text{Cl}_2$ /water) and the combined organic layer was dried ( $\text{MgSO}_4$ ) and concentrated to yield 2.46 g of a yellow solid. The detritylation was 15 carried out without DEAE Sephadex<sup>®</sup> purification.

**Detritylation:** The foam was stirred in 50 mL 3% DCA at room temperature, then pipetted onto a silica bed (approximately 120 mL) equilibrated with methylene chloride. The reaction mixture was eluted with 3% DCA then 100% methylene chloride through 10% methanol in methylene chloride. The appropriate fractions were combined and concentrated 20 to give 1.41 g of the detritylated tetramer (5'-HO-A-C-T-[3',3']-T), 72% overall yield for the two step process.

**Preparation of 5'-HO-T-A-C-T-[3',3']-T.** The detritylated tetramer 5'-HO-A-C-T-[3',3']-T (1.41 g, 0.8 mmol) was dried on high vacuum, then co-evaporated with dry methylene chloride and dissolved in 4.5 mL dry methylene chloride. Thymidine amidite 25 (0.78 g, 1.05 mmol) was added followed by tetrazole (0.5 M) in acetonitrile (8 mL, 4.02 mmol) and the reaction stirred under argon. After 2 hours, a 0.5 M iodine solution was added until the dark color persisted. The reaction was then concentrated and the gum was partitioned ( $\text{CH}_2\text{Cl}_2$ /water) and the combined organic layers were dried ( $\text{MgSO}_4$ ) and concentrated to yield 2.1 g of a yellow foam, which was analyzed by mass spectrometry and 30 reverse phase HPLC prior to elution through DEAE Sephadex<sup>®</sup>. Reverse phase HPLC analysis of the crude reaction mixture after oxidation showed the presence of pentamer, as

well as, unreacted tetramer (failure sequence) and hydrolyzed amidite monomer. ESMS (M-1) 803.74 x 3.

The yellow foam was dissolved in minimal methylene chloride and loaded onto a DEAE Sephadex® bed equilibrated with water and then methanol. The Sephadex® 5 was washed with methanol, methylene chloride and then acetonitrile. The appropriate fractions were combined and concentrated to give 1.48 g of material.

**Detritylation:** The material was stirred in 40 mL 3% DCA at room temperature, and then pipetted onto a silica bed equilibrated with methylene chloride. It was eluted with 3% DCA, followed by solutions of 100% methylene chloride through 20% methanol in methylene 10 chloride. The appropriate fractions were combined and concentrated to give 0.98 g of the detritylated pentamer (**5'-HO-T-A-C-T-[3',3']-T**), 64% overall yield for the two-step process. The <sup>31</sup>P NMR and its integration, are consistent with the product.

Preparation of 5'-HO-T-T-A-C-T-[3',3']-T. The detritylated pentamer **5'-HO-T-A-C-T-[3',3']-T** (0.96 g, 0.46 mmol) was dried under high vacuum, then co-evaporated 15 with dry methylene chloride and dissolved in 5 mL of dry methylene chloride. Thymidine amidite (0.44 g, 0.59 mmol) was added followed by tetrazole (0.5 M) in acetonitrile (4.5 mL, 2.27 mmol) and the reaction was stirred under argon. Since the solution was not homogenous, 2 mL of acetonitrile was added. After 2 hours, an additional 0.15 g of monomer was added and the reaction was stirred overnight. A 0.5 M iodine solution was 20 added, followed by 5% NaHSO<sub>3</sub>, changing the color from brown to yellow. The concentrated reaction was partitioned (CH<sub>2</sub>Cl<sub>2</sub>/water) and the organic layer was dried (MgSO<sub>4</sub>) and concentrated to yield 1.61 g of a yellow solid which was analyzed by MS. ESMS (M-1) 1384.01 x 2.

The crude reaction mixture (1.48 g) was absorbed onto C18 resin and loaded 25 onto a bed of C18 resin (approximately 125 g) which had been equilibrated with acetonitrile, followed by 70% water/acetonitrile. The resin was first washed with 1:1 water:acetonitrile to elute the monomer, followed by acetonitrile and methylene chloride to elute the hexamer. The appropriate fractions were combined and concentrated to give 0.83 g, (66% yield) of a solid.

30 **Detritylation:** The solid was stirred in 20 mL of 3% DCA at room temperature. Trihexylsilane (2 mL) was added and stirring was continued. Upon addition of hexane a

solid formed which was washed with hexane/ether to give 0.5 g of pink solid. The  $^{31}\text{P}$  NMR and its integration, are consistent with the product (5'-HO-T-T-A-C-T-[3',3']-T).

Dowex C1-form can be used to remove residual DCA from a solid sample. For example, a T-A phosphoramidite dimer was found by NMR to contain approximately 5 1.2 equivalents of DCA following detritylation and hexane precipitation. A sample of this dimer (0.3 g) was dissolved in acetonitrile (5 mL) and loaded onto a column of Dowex C1-form (15 g) which had been pre-equilibrated with acetonitrile. The liquid was eluted dropwise and the column was then washed with 35 mL of acetonitrile and concentrated to yield 0.26 g of a white foam. A sample checked by NMR shows approximately 95% 10 reduction of acid.

**Example 6. Automation of PASS using hydrophobic affinity to capture the product**

After the coupling reaction, e.g., the reaction of 12 with 17 in Example 2 (Scheme 5), the reaction mixture is pumped into extraction vessel 112, through inlet port 15 128 (Figure 5). Triethylammonium bicarbonate buffer (TBK) (0.05 M) and  $\text{CH}_2\text{Cl}_2$  are added to the extraction vessel through inlet port 130, and the mixture is stirred. The layers are allowed to separate. After separation, valve 120 opens and the methylene chloride layer passes through conductivity meter 136, and onto a DEAE Sephadex<sup>®</sup> plug 114. A rise in conductivity indicates that the  $\text{CH}_2\text{Cl}_2$  has completely passed through the conductivity meter 20 and the aqueous layer has now entered the meter. At this time, valve 120 automatically switches to divert the aqueous layer away from the DEAE Sephadex<sup>®</sup> plug. The organic layer is pushed through the DEAE Sephadex<sup>®</sup> plug with argon which enters the chamber through inlet port 140. The DEAE Sephadex<sup>®</sup> plug is then washed with  $\text{CH}_2\text{Cl}_2$ , which is added through inlet port 142, controlled by valve 122. The  $\text{CH}_2\text{Cl}_2$  effluent, which contains 25 the oligonucleotide product and unreacted oligonucleotide starting material (failure sequence), is collected through outlet port 144, controlled by valve 124. Upon complete elution of  $\text{CH}_2\text{Cl}_2$ , the unreacted phosphoramidite monomer, which has been retained on the Sephadex<sup>®</sup> plug, is eluted with the 1 M TBK. The Sephadex<sup>®</sup> plug is then re-equilibrated with  $\text{CH}_2\text{Cl}_2$ . 30 The  $\text{CH}_2\text{Cl}_2$  eluent, is then passed through a reverse phase resin to separate the coupled product from the failure sequence. The coupled product, which has a DMT

group attached to its 5'-end, is retained on the resin, and the failure sequence is eluted from the chamber. The resin is then washed with, acidic dichloroacetic acid (3% in CH<sub>2</sub>Cl<sub>2</sub>), which cleaves the DMT protecting group and releases the coupled product from the chamber. The coupled product is eluted into a pH buffered solution to prevent 5 decomposition due to excessive exposure to acid. The eluent is concentrated and the coupled product used as the starting material in the next reaction cycle.

**Example 7. Preparation of a 3'-PEG anchored 15mer DNA by solution phase synthesis**

An oligonucleotide of sequence 5'-CTAACCGTAATGG-[3',3']-T-T-3' (SEQ 10 ID NO:1) was prepared by liquid phase synthesis, using polyethylene glycol (PEG) of molecular weight 20,000 as the 3'-terminal modification. Polyethylene glycol allows facile precipitation of the growing oligonucleotide chain during the individual steps. This example outlines the basic steps required for solution phase synthesis without the incorporation of the capture of the oligonucleotide coupling product onto a resin as in a 15 typical PASS cycle. Thus, this example demonstrates the impact on efficiency and product purity, that product capture provides as envisioned in PASS. With such product capture at each monomer addition cycle, the cumbersome precipitations from diethyl ether are no longer necessary. In addition, because failure sequences are removed at each monomer addition cycle, the anion exchange chromatogram of the product obtained by PASS is 20 expected to only show a single product peak, rather than the multiple peaks seen in Figure 6.

This example provides the general procedures followed for each monomer addition cycle for the preparation of a 3'-PEG anchored oligonucleotide by solution phase synthesis without the incorporation of product capture as a means to separate product from failure sequence. All of the following reactions were performed in a one-neck flask with a 25 self-sealing septum at room temperature. Disposable plastic syringes were used.

Detritylation 5'-DMT-nucleoside 3'-O-PEG (5.0 g) (20k, loading: 45 µmol/g) was dissolved in 50 mL of a mixture of dichloroacetic acid (DCA) and trihexylsilane (6.4 mL, 80 equivalents) in CH<sub>2</sub>Cl<sub>2</sub>. After 9 minutes the detritylated 5'-HO-nucleoside 3'-O-PEG was precipitated with ether (2x), washed, filtered and dried under vacuum.

30 **Coupling reaction:** The 5'-HO-nucleoside 3'-O-PEG was coevaporated 3 times with 20 mL of anhydrous acetonitrile and dried under high vacuum for 30 minutes. The flask was

flushed with argon and closed to the outer atmosphere. Through the septa was injected: 50 mL of anhydrous acetonitrile to dissolve the 5'-HO-nucleoside 3'-O-PEG, 4.5 mL (0.1 M, 2.0 equivalents) of amidite in anhydrous acetonitrile and 1.4 mL (1.0 M, 6.0 equivalents) of DCI in acetonitrile. The solution was stirred under argon for 25 minutes, then precipitated

5 with ether and dried by coevaporation with 20 mL of anhydrous acetonitrile.

**Oxidation:** The precipitate was dissolved in 50 mL of anhydrous acetonitrile, and 8 mL (0.1 M) of iodobenzene diacetate in acetonitrile was injected and the reaction mixture was stirred for 8 minutes.

**Capping reaction:** Acetic anhydride (6 mL), 2,6-lutidine (6 mL) and N-methylimidazole (6

10 mL) were simultaneously injected to the above solution and the reaction mixture was stirred for another 5 minutes. The capped oligonucleotide-PEG polymer was precipitated from ether as described above in the detritylation procedure.

**Crystallization:** The capped oligonucleotide-PEG polymer was purified by crystallization from 500 mL of absolute ethanol (100 mL/g) at 60°C.

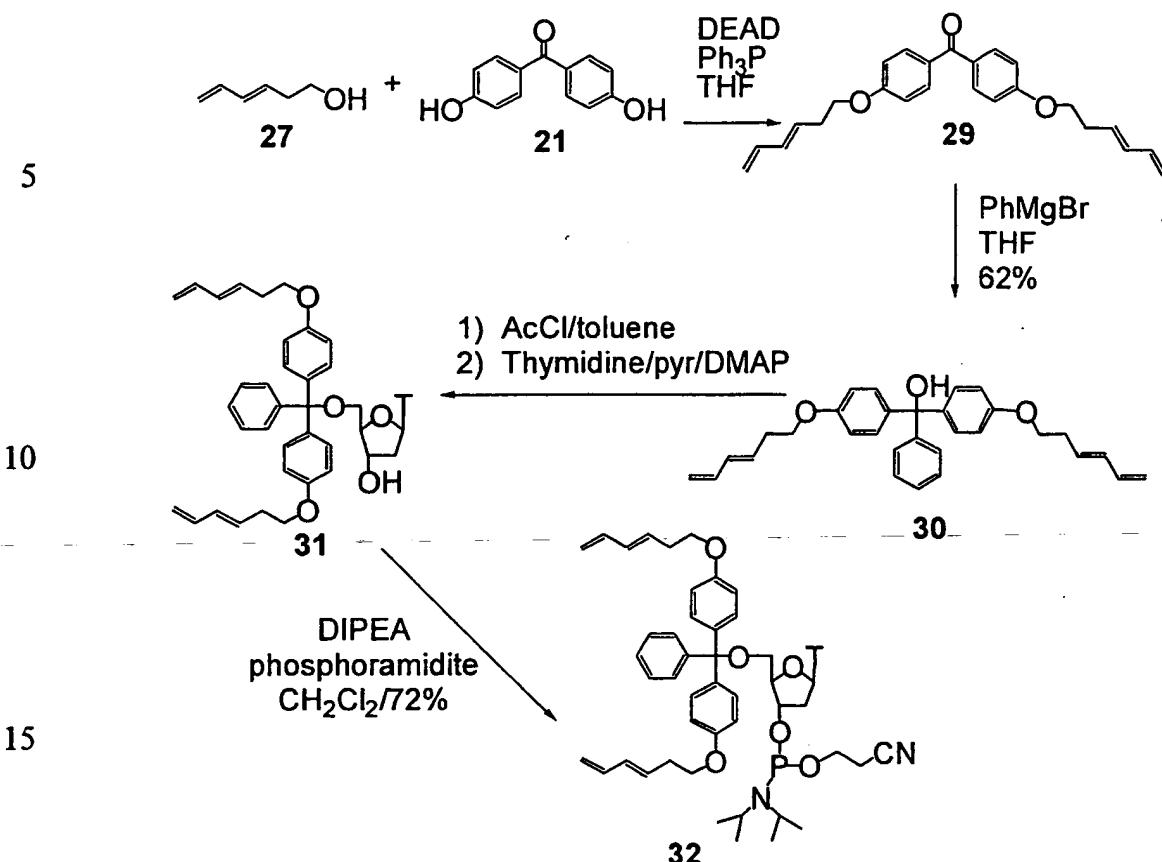
15 The monomer addition cycle protocol is summarized in Table 2. The stepwise coupling efficiency for the preparation of the 3'-terminal 10 base fragment (10mer) (CGTAATGG-[3',3']-T-T) of oligonucleotide (SEQ ID NO:2), is shown in Table 3. The anion exchange HPLC chromatogram of the crude 15mer (5'-CTAACCGTAATGG-[3',3']-T-T-3' (SEQ ID NO:1) after cleavage from the PEG and deprotection is shown in

20 Figure 6.

**Example 8. Preparation of diene modified trityl alcohols**

Example 8 (Schemes 7 and 8) describes the synthesis of various diene modified trityl alcohols including a 5'-O-(4,4'-di-3,5-hexadienoxytrityl) thymidine 3'-  
25 phosphoramidite monomer 32.

### SCHEME 7



Preparation of 4,4'-di-3,5-hexadienoxybenzophenone (29). To a solution of 3,5-hexadienol (27) (13.7 g, 140 mmol) (Martin *et al.* (1980) J. Am. Chem. Soc. **102**:5274-5279) in anhydrous THF (335 mL) was added 4,4'-dihydroxybenzophenone (21) (10.0 g, 46.7 mmol) and triphenylphosphine (36.7 g, 140 mmol) followed by the slow addition of diethylazodicarbonate (DEAD) (22.0 mL, 140 mmol). The reaction mixture was stirred under argon overnight and then evaporated to dryness under vacuum. A precipitation from dichloromethane-hexane was carried out to remove residual reagents. The filtrate was concentrated *in vacuo* and purified by column chromatography (silica gel; hexane/CH<sub>2</sub>Cl<sub>2</sub>, 3/2) to afford an impure product which was triturated (Et<sub>2</sub>O/hexane, 1/1) to give 7.12 grams of compound 29. Further purification of the filtrate by column chromatography (silica gel; hexane/CH<sub>2</sub>Cl<sub>2</sub>, 3/2) afforded an additional 5.96 grams of 29 to give a total of 13.08 g (75%) of compound 29 as a white solid. <sup>1</sup>H NMR (300 MHz, DMSO-d<sub>6</sub>) δ 2.50-2.64 (m, 4H), 4.16 (t, J=6.5 Hz, 4H), 5.05 (d, J=10.1 Hz, 2H), 5.18 (d, J=15.7 Hz, 2H), 5.77-5.92 (m, 2H), 6.17-6.47 (m, 4H), 7.10 (d, J=8.6 Hz, 4H), 7.72 (d, J=8.7 Hz, 4H).

Preparation of 4,4'-di-3,5-hexadienoxytrityl alcohol (30). Compound 29

(5.96 g, 15.91 mmol) was dissolved in anhydrous THF (133 mL) with slight heating.

Phenylmagnesium bromide (32 mL of a 1.0 M solution in THF, 32 mmol) was added to the solution and the mixture was stirred at room temperature under argon for 5 hours and

5 evaporated to dryness under vacuum. The residue was redissolved in dichloromethane and washed with a saturated solution of ammonium chloride, followed by water. The organic phase was dried ( $\text{MgSO}_4$ ), concentrated *in vacuo*, and purified by column chromatography (silica gel; hexane/ $\text{CH}_2\text{Cl}_2$ , 1/9) to yield 4.45 grams (62%) of compound 30 as a yellow oil.

$^1\text{H}$  NMR (300 MHz,  $\text{DMSO-d}_6$ )  $\delta$  2.45-2.56 (m, 4H), 3.98 (t,  $J=6.6$  Hz, 4H), 5.01 (dd,

10  $J=1.5, 9.9$  Hz, 2H), 5.14 (dd,  $J=1.5, 16.5$  Hz, 2H), 5.73-5.87 (m, 2H), 6.12-6.41 (m, 4H), 6.25 (s, 1H), 6.84 (d,  $J=6.9$  Hz, 4H), 7.06 (d,  $J=7.8$  Hz, 4H), 7.15-7.33 (m, 5H).

Preparation of 5'-O-(4,4'-di-3,5-hexadienoxytrityl)thymidine (5'-DHDTO-dT) (31).

Compound 30 (3.5 grams, 7.73 mmol) was coevaporated with toluene (2x) and then dissolved in anhydrous toluene (85 mL). Acetyl chloride (33 mL, 464 mmol) was added to

15 the solution and the reaction mixture was heated to reflux and stirred under argon. After 4 hours the reaction mixture was concentrated *in vacuo* and the crude product was coevaporated with pyridine and then dissolved in anhydrous pyridine (42 mL). Thymidine (1.5 grams, 6.18 mmol), which had been coevaporated with pyridine and dissolved in anhydrous pyridine (42 mL), was then added to the solution containing the crude product. A

20 catalytic amount of dimethylaminopyrimidine (DMAP) was added and the reaction mixture was stirred under argon overnight and the solvent was evaporated. The residue was redissolved in dichloromethane and washed with a 5% aqueous solution of sodium bicarbonate followed by water. The organic phase was dried ( $\text{MgSO}_4$ ), evaporated and purified by column chromatography (silica gel;  $\text{EtOAc/hexane}$ , 1/1) to afford 3.53 grams

25 (84%) of compound 31 as an off-white solid.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  1.47 (s, 3H), 2.22-2.46 (m, 2H), 2.50-2.63 (m, 4H), 3.35-3.58 (m, 2H), 3.85-4.09 (m, 5H), 4.51-4.60 (m, 1H), 5.02 (dd,  $J=1.5, 10.4$  Hz, 2H), 5.14 (dd,  $J=1.5, 17.3$  Hz, 2H), 5.68-5.83 (m, 2H), 6.12-6.45 (m, 5H), 6.82 (d,  $J=9.0$  Hz, 4H), 7.18-7.46 (m, 9H), 7.58 (s, 1H), 8.44 (s, 1H); Anal.

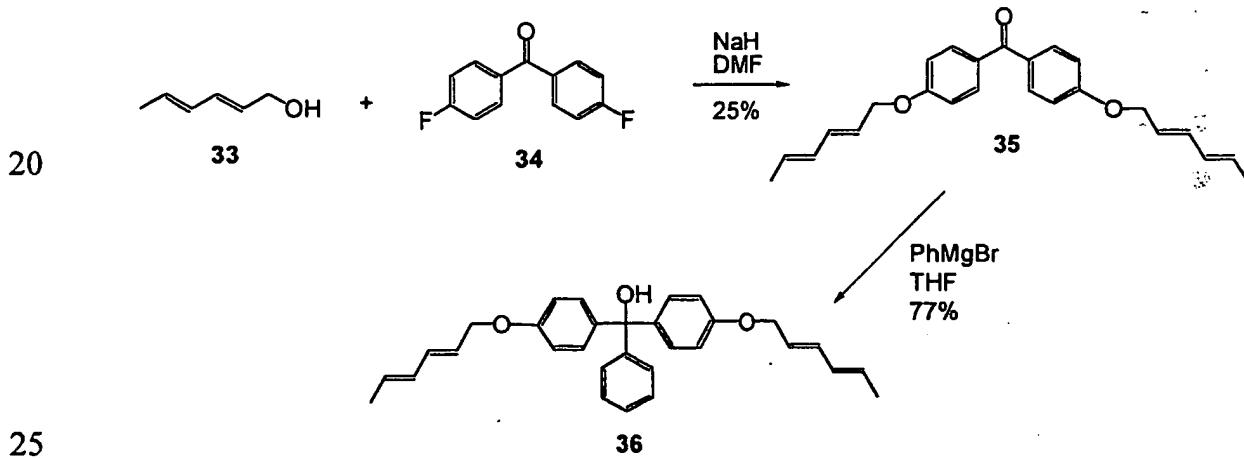
Calcd for  $\text{C}_{41}\text{H}_{44}\text{N}_2\text{O}_7\cdot 2\text{H}_2\text{O}$  (712.8384): C, 69.08; H, 6.79; H, 3.93. Found: C, 69.34; H,

30 6.44; N, 3.91.

Preparation of 5'-O-(4,4'-di-3,5-hexadienoxytrityl)thymidine 3'-phosphoramidite (32).

Compound 31 (3.0 grams, 4.43 mmol) was dissolved in anhydrous dichloromethane and diisopropylethylamine (2.7 mL; 15.5 mmol) was added. The solution was cooled to 0°C and 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (2.0 mL, 8.86 mmol) was added. The reaction mixture was allowed to warm to room temperature with stirring under argon. After 4 hours the solution was diluted with dichloromethane and washed with a 5% aqueous solution of sodium bicarbonate (2x). The organic phase was dried ( $\text{MgSO}_4$ ), concentrated *in vacuo*, and purified by column chromatography (silica gel; EtOAc/hexane, 3/7) to afford 2.8 grams (72%) of compound 32 as a fluffy white solid.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  1.01-1.20 (m, 12H), 1.41 (s, 3H), 2.25-2.67 (m, 8H), 3.26-4.22 (m, 11H), 4.60-4.65 (m, 1H), 5.02 (dd,  $J=1.5, 10.4$  Hz, 2H), 5.14 (dd,  $J=1.5, 17.3$  Hz, 2H), 5.69-5.84 (m, 2H), 6.11-6.48 (m, 5H), 6.82 (dd,  $J=3.2, 8.9$  Hz, 4H), 7.16-7.43 (m, 9H), 7.62 (d,  $J=15.2$  Hz, 1H), 8.05 (bs, 1H);  $^{31}\text{P}$  NMR (300 MHz,  $\text{DMSO-d}_6$ ) 152.9, 152.4; Anal. Calcd for  $\text{C}_{50}\text{H}_{61}\text{N}_4\text{O}_8\text{P}_1$  (877.0276): C, 68.48; H, 7.01; N, 6.39. Found: C, 68.48; H, 7.22; N, 6.33.

## SCHEME 8



Preparation of 4,4'-di-2,4-hexadienoxybenzophenone (35). 4,4'-

Difluorobenzophenone (34) (4.8 grams, 22 mmol) was dissolved in anhydrous DMF (1 liter). NaH (95%; 5.6 grams, 220 mmol) was added and the solution was cooled to 0°C. 2,4-Hexadienol (5.8 mL, 51 mmol) was slowly added to the solution and the reaction mixture was allowed to warm to room temperature with stirring under argon overnight. The reaction mixture was concentrated *in vacuo*, dissolved in dichloromethane, and washed with

water. The organic phase was dried ( $\text{MgSO}_4$ ) and concentrated and purified by column chromatography (silica gel; hexane/ $\text{CH}_2\text{Cl}_2$ , 1/3) to afford 2.07 grams (25%) of compound 35 as a white solid.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-d}_6$ )  $\delta$  1.73 (d,  $J=6.6$  Hz, 6H), 4.68 (d,  $J=6.0$  Hz, 4H), 5.69-5.84 (m, 4H), 6.05-6.19 (m, 2H), 6.31-6.45 (m, 2H), 7.08 (d,  $J=9.0$  Hz, 4H), 7.68 (d,  $J=10.2$  Hz, 4H).

Preparation of 4,4'-di-2,4-hexadienoxytrityl alcohol (36). Compound 35 (2.0 grams) was dissolved in THF (45 mL) and phenylmagnesium bromide (1.0 M solution in THF; 10.6 mL, 10.6 mmol) was added to the solution. The reaction mixture was stirred at room temperature for 3 hours, and evaporated to dryness under vacuum. The residue was 10 redissolved in dichloromethane and washed with a saturated solution of ammonium chloride, followed by water. The organic phase was dried ( $\text{MgSO}_4$ ), concentrated *in vacuo* and purified column chromatography (silica gel; hexane/ $\text{CH}_2\text{Cl}_2$ , 1/9) to afford 1.84 grams (77%) of compound 36 as a pale yellow solid.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-d}_6$ )  $\delta$  1.73 (d,  $J=6.9$  Hz, 6H), 4.54 (d,  $J=6.0$  Hz, 4H), 5.68-5.80 (m, 4H), 6.02, 6.11 (m, 2H), 6.20-6.37 (m, 15 2H), 6.85 (d,  $J=6.9$  Hz, 4H), 7.05 (d,  $J=7.0$  Hz, 4H), 7.14-7.32 (m, 5H); Anal. Calcd for  $\text{C}_{31}\text{H}_{32}\text{O}_3$  (452.5920): C, 82.27; H, 7.13; Found: C, 82.30; H, 7.11.

The 5'-di-(2,4-hexadienoxy)tritylthymidine phosphoramidite monomer can be prepared from compound 36 using the same procedure described above for the preparation of the 5'-O-(4,4'-di-3,5-hexadienoxytrityl)thymidine phosphoramidite (32).

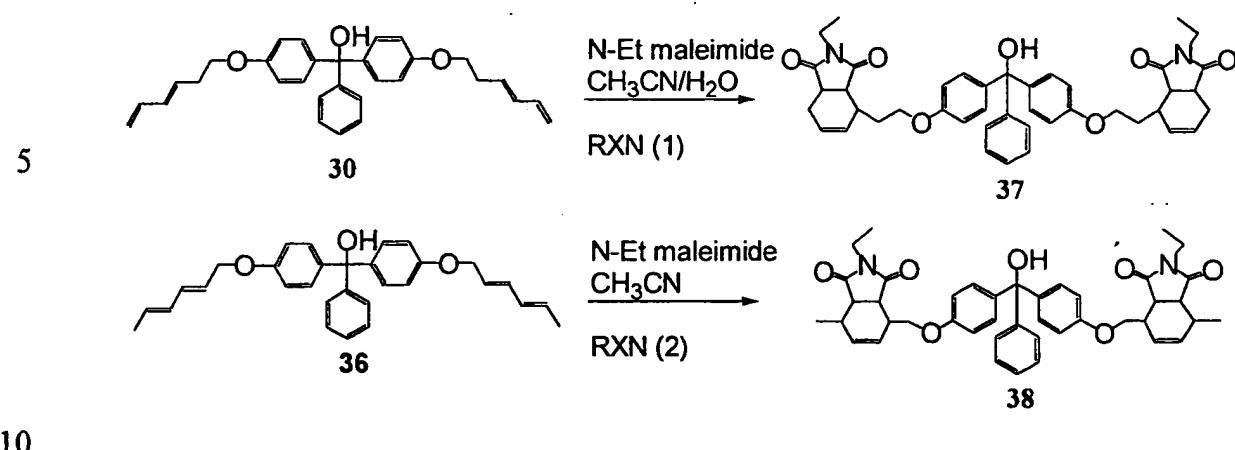
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Example 9. Diels-Alder cycloaddition of diene substituted trityl alcohols with N-ethylmaleimide

Example 9 (Scheme 9) describes the Diels-Alder reaction of diene substituted trityl alcohols --4,4'-di-3,5-hexadienoxytrityl alcohol (30) and 4,4'-di-2,4-25 hexadienoxytrityl alcohol (36)-- with N-ethyl maleimide (Reactions 1 and 2 respectively). Table 4 sets forth the reaction rates for these two reactions under various reaction conditions.

30

## SCHEME 9

Diels-Alder reaction of 3,5-hexadienoxytrityl alcohol (30) - Reaction 1.

Compound 30 (50 mg, 0.11 mmol) was dissolved in acetonitrile (0.75 mL) and water (0.75 mL). N-ethyl maleimide (N-Et maleimide) (138 mg, 1.1 mmol) was added and the reaction mixture was stirred at room temperature. After 3 hours <sup>1</sup>H NMR analysis of the crude reaction mixture showed that the reaction had gone to completion. The reaction mixture was concentrated and loaded onto a silica gel plug pre-equilibrated with dichloromethane. The excess N-ethyl maleimide was washed off with dichloromethane and the product was eluted with 10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>. The solvent was concentrated under reduced pressure to afford 38 mg (59%) of compound 37. <sup>1</sup>H NMR (300 MHz, DMSO-d<sub>6</sub>) δ 0.97 (t, J=7.2 Hz, 6H), 2.02-2.19 (m, 4H), 2.20-2.34 (m, 2H), 2.42-2.53 (m, 4H), 3.13-3.24 (m, 4H), 3.28-3.39 (m, 4H), 4.11 (t, J=6.3 Hz, 4H), 5.70-5.86 (m, 4H), 6.22 (s, 1H), 6.87 (d, J=9.0 Hz, 4H), 7.07 (d, J=9.0 Hz, 4H), 7.15-7.24 (m, 5H).

Diels-Alder reaction of 2,4-hexadienoxytrityl alcohol (36) - Reaction 2.

Compound 36 (60 mg, 0.13 mmol) was dissolved in acetonitrile (2.0 mL). N-ethyl maleimide (166 mg, 1.3 mmol) was added and the reaction mixture was stirred at room temperature. After 24 hours <sup>1</sup>H NMR analysis of the crude reaction mixture showed the reaction had gone to completion. The reaction mixture was concentrated and loaded onto a silica gel plug pre-equilibrated with dichloromethane. The excess N-ethyl maleimide was washed off with dichloromethane and the product was eluted with 10% MeOH/CH<sub>2</sub>Cl<sub>2</sub> and concentrated under reduced pressure to yield 50 mg (54%) of compound 38. <sup>1</sup>H NMR (300 MHz, DMSO-d<sub>6</sub>) δ 0.95 (t, J=7.1 Hz, 6H), 1.32 (d, J=7.2 Hz, 6H), 2.48 (bs, 2H), 2.74 (bs,

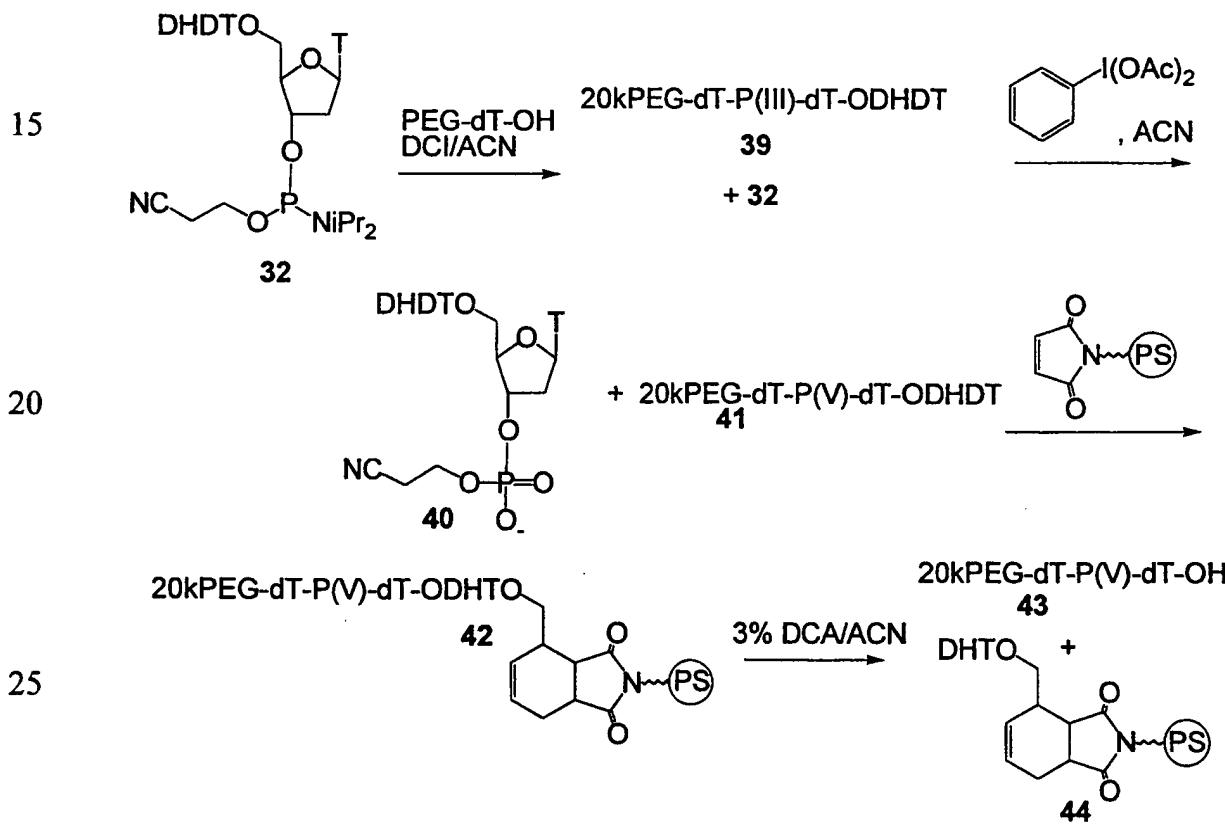
2H), 3.05-3.46 (m, 8H), 4.26 (t,  $J=8.4$  Hz, 2H), 4.50 (t, 8.4 Hz, 2H), 5.67-5.86 (m, 4H), 6.27 (s, 1H), 6.88 (d,  $J=8.7$  Hz, 4H), 7.11 (d,  $J=8.7$  Hz, 4H), 7.16-7.35 (m, 5H); Anal. Calcd for  $C_{43}H_{46}N_2O_7\cdot 2H_2O$  (738.8762): C, 69.90; H, 6.82; N, 3.79. Found: C, 71.16; H, 6.71; N, 3.92.

5

**Example 10. Preparation of 3'-PEG-linked oligonucleotides using product capture by Diels-Alder cycloaddition**

Example 10 (Scheme 10) provide the general procedures to be followed for each monomer addition cycle, for the preparation of a 3'-PEG anchored oligonucleotide by 10 solution phase synthesis using the Diels-Alder cycloaddition reaction for the covalent capture of the oligonucleotide product.

SCHEME 10



**Coupling Reacti n:** PEG-dT-OH (20k, 2.36 g, 0.11 mmol, loading: 46  $\mu$ mol/g) was 30 dissolved in 20 mL of dry acetonitrile ( $CH_3CN$ ) under an atmosphere of dry argon. To this solution was added 5'-O-(4,4'-di-3,5-hexadienoxytrityl)thymidine 3'-phosphoramidite (32)

(140 mg, 0.16 mmol), followed by DCI in CH<sub>3</sub>CN (0.65 mL, 1.0 M, 6.0 equivalents). The reaction was stirred under an atmosphere of dry argon for 25 minutes, after which 350 mL of dry Et<sub>2</sub>O was added to precipitate out the 20k-PEG containing material. The solids were filtered and washed with Et<sub>2</sub>O (2x100 mL) and dried under vacuum for 1 hour to yield 2.3 g

5 of a white solid (98% mass yield).

**Oxidation:** The white solid, which contains coupled product 39, unreacted phosphoramidite 32 and unreacted PEG-dT-OH, is redissolved in 20 mL CH<sub>2</sub>Cl<sub>2</sub> and oxidized with iodobenzene diacetate in CH<sub>3</sub>CN (8.5 mL, 0.1 M, 0.27 g). After stirring for 8 minutes, the reaction mixture contains unreacted PEG-dT-OH, oxidized amidite monomer

10 40 and the oxidized oligomer 41. The reaction mixture is then treated with 350 mL of dry Et<sub>2</sub>O to precipitate the 20k-PEG containing material and the solids are filtered and washed with 2x100 mL Et<sub>2</sub>O. After drying under vacuum for 1 hour, a white solid is isolated which contain the unreacted PEG-dT-OH and the oligomer 41.

**Diels-Alder Cycloaddition:** The solids are redissolved in 20 mL of 50% H<sub>2</sub>O/CH<sub>3</sub>CN and

15 loaded onto 1.2 g (10 equivalents based on a maleimide loading of 0.4 mmol maleimide/g resin) of maleimide-functionalized polystyrene, which has been prewetted with 5 mL of 50% H<sub>2</sub>O/CH<sub>3</sub>CN. The reaction is warmed to 45°C for 1 hour under an atmosphere of argon. It is expected that reverse-phase HPLC analysis of the supernatant liquid will reveal that the 5'-protected oligomer 41 has been completely consumed. The maleimide-

20 derivatized polystyrene 42 can then be filtered and washed with 10 mL of 50% H<sub>2</sub>O/CH<sub>3</sub>CN, to yield 3.5 g of 3'-PEG-5'-DHDT Diels-Alder conjugate oligomer (42) as a solid resin.

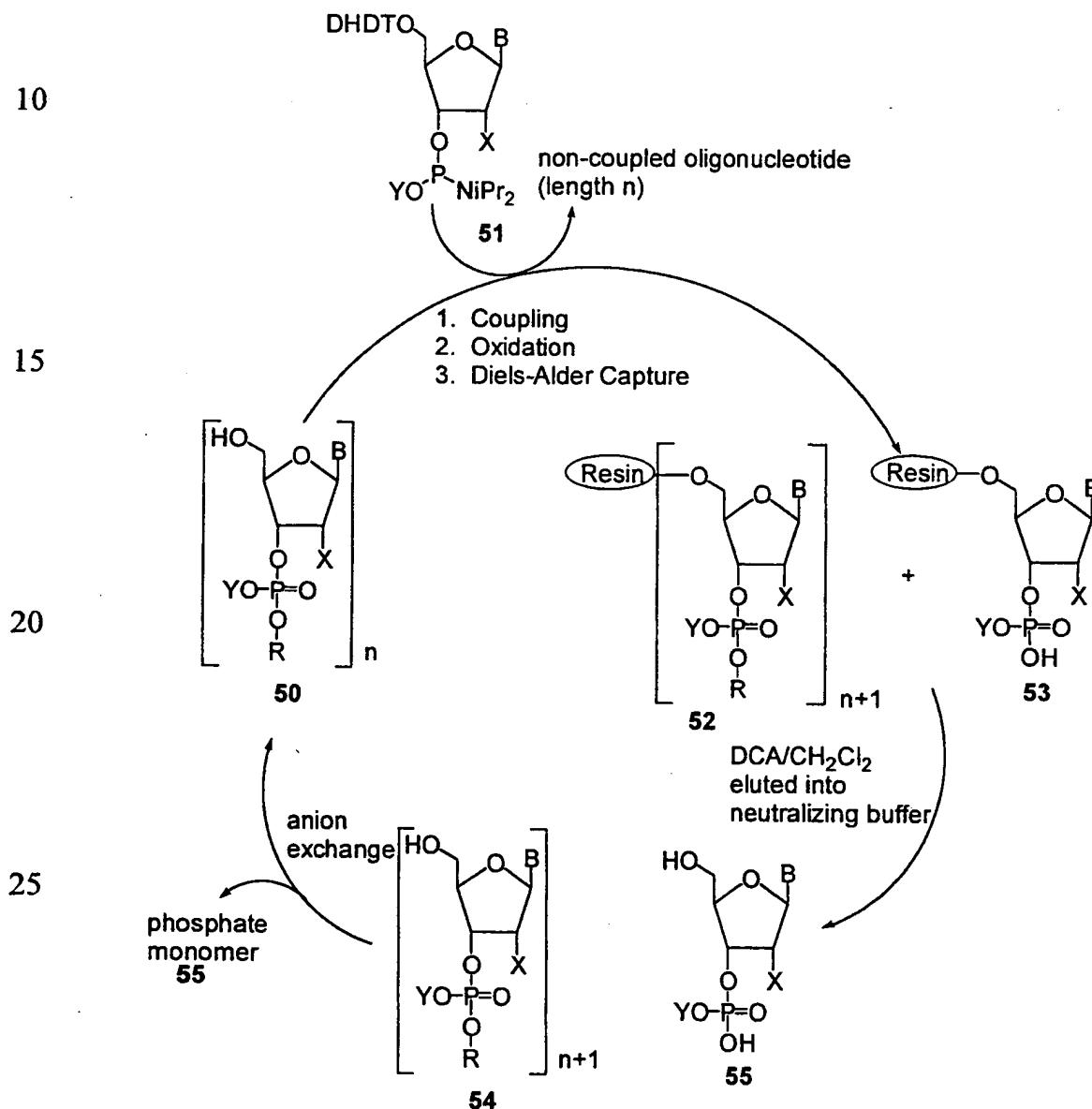
**Detritylation/Oligonucleotide Release:** It is anticipated that the 3.5 g of Diels-Alder conjugate resin 42 (loading: 75  $\mu$ mol/g) can be suspended in 20 mL of CH<sub>2</sub>Cl<sub>2</sub>. To this

25 suspension is added a mixture of DCA and trihexylsilane (6.4 mL, 80 equivalents) in CH<sub>2</sub>Cl<sub>2</sub>. After 9 minutes the polystyrene-maleimide resin (44) is removed via filtration. The PEG-nucleoside (43) is then precipitated twice with Et<sub>2</sub>O (500 mL), washed, filtered and dried under vacuum. The resultant PEG-nucleoside is deprotected at the 5'-position and is ready for the next coupling reaction of the sequence.

Example 11. Preparation of non-PEG derivatized oligonucleotides by Diels-Alder product capture

Scheme 12 illustrates a general reaction scheme for the preparation of a non-PEG derivatized oligonucleotide by Diels-Alder product capture using a 5'-O-(4,4'-di-3,5-5 hexadienoxytrityl)nucleoside (5'-O-DHDT-nucleoside) as the diene and a maleimide substituted solid support as the dienophile.

SCHEME 12



30 DHDT: 4,4'-di-3,5-hexadienoxytrityl  
 Resin: Maleimide-derivatized solid support (CPG, silica, cellulose, HLP, etc)  
 X: any suitably protected 2'-substituent

Y: phosphate protecting group

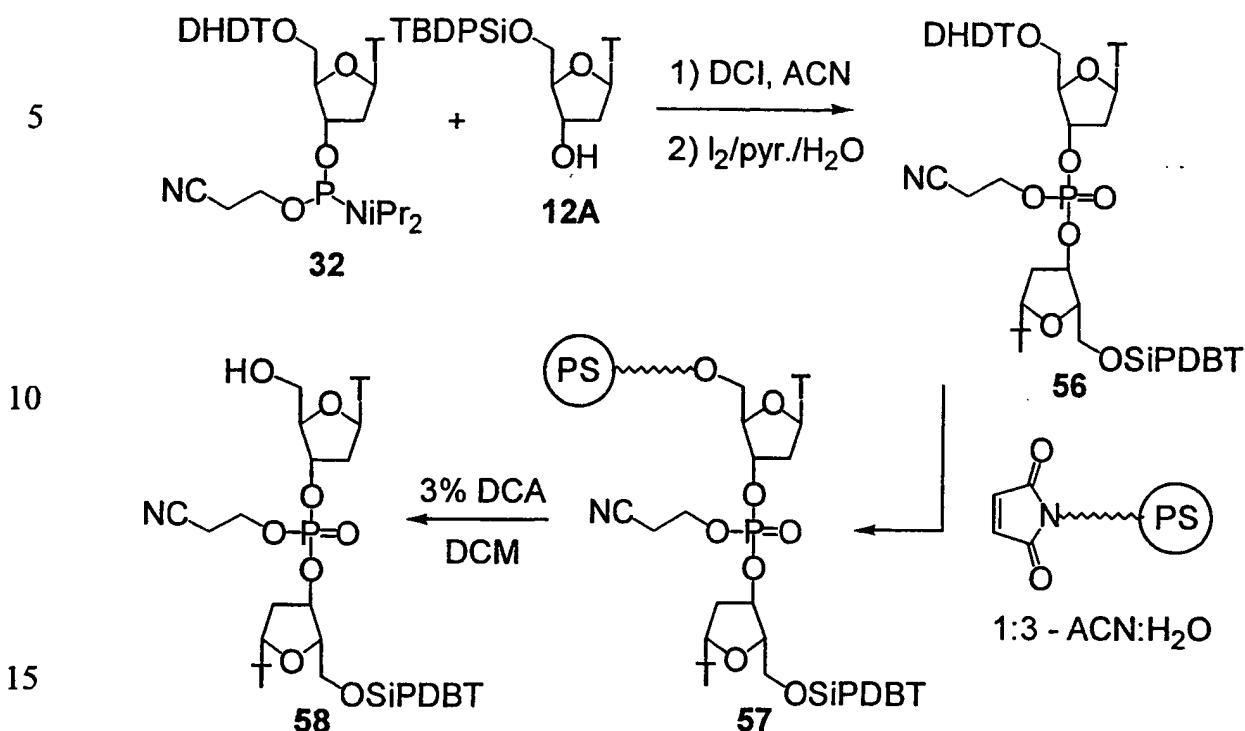
B: suitably protected, modified, or derivatized nucleobase

R: oligonucleotide or 3'-blocking group

5 **Coupling/Oxidation/Capture Sequence:** In CH<sub>3</sub>CN, the appropriate 3'-blocked oligonucleotide of length n (50) is coupled with 2.0 equivalents of the amidite monomer 51 by treatment with a 1.0 M solution of DCI in CH<sub>3</sub>CN. The 3'-blocking group can be a lipid or polysaccharide, or a more traditional solution-phase blocking group such as acetyl, pyranyl, or silyl group, such as tert-butyldiphenylsilyl ether. The coupling reaction takes  
10 less than 25 minutes and is monitored by TLC. Upon completion of the coupling reaction, the solution is treated directly with 8.0 equivalents of iodobenzene diacetate as a 0.1 M solution in CH<sub>3</sub>CN. The oxidation sequence is complete within 8 minutes and the crude reaction mixture is applied directly to the solid support bearing the dienophile. The Diels-Alder cycloaddition reaction is accelerated by utilizing a solvent of 1:1 CH<sub>3</sub>CN:H<sub>2</sub>O. The  
15 oligonucleotide, now covalently bound to the solid support 52, is easily separated from the unreacted starting oligonucleotide 50 (failure sequence) and reagents via simple filtration and washing of the resin beads. The amidite monomer 51 which also has a 5'-DHD'T group is also bound to the resin (53).

**Detritylation/Release Sequence:** The washed and dried resin, bearing the covalently  
20 bound oligonucleotide (52), as well as, unreacted monomer phosphate (53), is washed with a solution of 3% DCA/CH<sub>2</sub>Cl<sub>2</sub>, eluting into a neutralizing buffer to prevent acid-mediated decomposition of the oligonucleotide chain. The released oligomer (54) and monomer phosphate (55) are separated from one another via aqueous extraction. The product oligonucleotide in the organic phase is dried and exchanged into acetonitrile by  
25 ultrafiltration.

**Example 12. Preparation of a dimer using product capture by Diels-Alder cycloaddition**



**Preparation of 5'-DHDTO-T-[3',3']-T-OSiPDBT-5' (56).** 5'-TBDPSiO-dT-3'-OH (12A) (0.21 g, 0.43 mmol) was dissolved in 10 mL acetonitrile. 5'-DHDTO-dT phosphoramidite (32) (0.5 g, 0.52 mmol) was added to this solution followed by 3.0 mL of 1.0 M DCI in acetonitrile (3.0 mmol). This solution was stirred under argon for 20 minutes, at which time 11 mL of a solution of 0.2 M I<sub>2</sub> in pyridine/water was added. The oxidation reaction was allowed to proceed for 5 minutes and was filtered (4x) through DEAE Sephadex<sup>®</sup> to remove most of the yellow color. A yellow solid 56 (0.23 g) was isolated.

**Product Capture:** The Diels-Alder capture reaction was performed with variation in the amount of polystyrene supported maleimide (PS-M) used, as follows: 10 eq, 5 eq, 2.5 eq, 1 eq. The procedure below for all of the reactions was as follows. The [3',3']-dT-dT-OTDHD dimer (11  $\mu$ mol) (56) was dissolved in 400  $\mu$ L of acetonitrile. This solution was added to a suspension of PS-M in 1.0 mL of 3/1 CH<sub>3</sub>CN/water and then warmed to 65 °C. The course of the reaction was monitored by TLC (2/1 EtOAc/hexanes), by the disappearance of the reactant at  $R_f$  = 0.15, and via HPLC analysis (C18, 4.6 X 100 mm, Buffer A: 100 mM triethylammonium acetate pH 7.5, Buffer B: acetonitrile, 0 to 80% B over 2.5 minutes). The

% reaction was determined by comparison to the initial ratio of dimerized material (2.65 min) to unreacted 5'-TBDPSiO-dT-3'-OH monomer (12A) (1.71 min). (See Figure 7). It is interesting to note that the lines drawn for 2.5 equiv., 1.0 equiv. and control (No PS-M) all show reaction (disappearance of dimer) occurring after 4 hours. The reaction is not a Diels-Alder capture, but is rather decomposition of the dimer by what is believed to be hydrolysis. A new material at 1.47 minutes and 2.30 minutes appears in the HPLC traces. This material may be 5'-TBDPSiO-dT-3'-phosphate (1.47 minutes) and 5'-DHDTO-dT-3'-phosphate. Since 5'-TBDPSiO-dT-3'-OH is also expected to be produced in the course of the hydrolysis reaction, the relative rates of Diels-Alder capture cannot be directly obtained from these traces as the internal standard is not appropriate in the cases where hydrolysis is evident. Hydrolysis can be corrected for by adjusting to the amount of 5'-TBDPSiO-dT-3'-phosphate evident in the later traces. This process is not significant in the case of 5.0 equiv. and 10.0 equivalents.

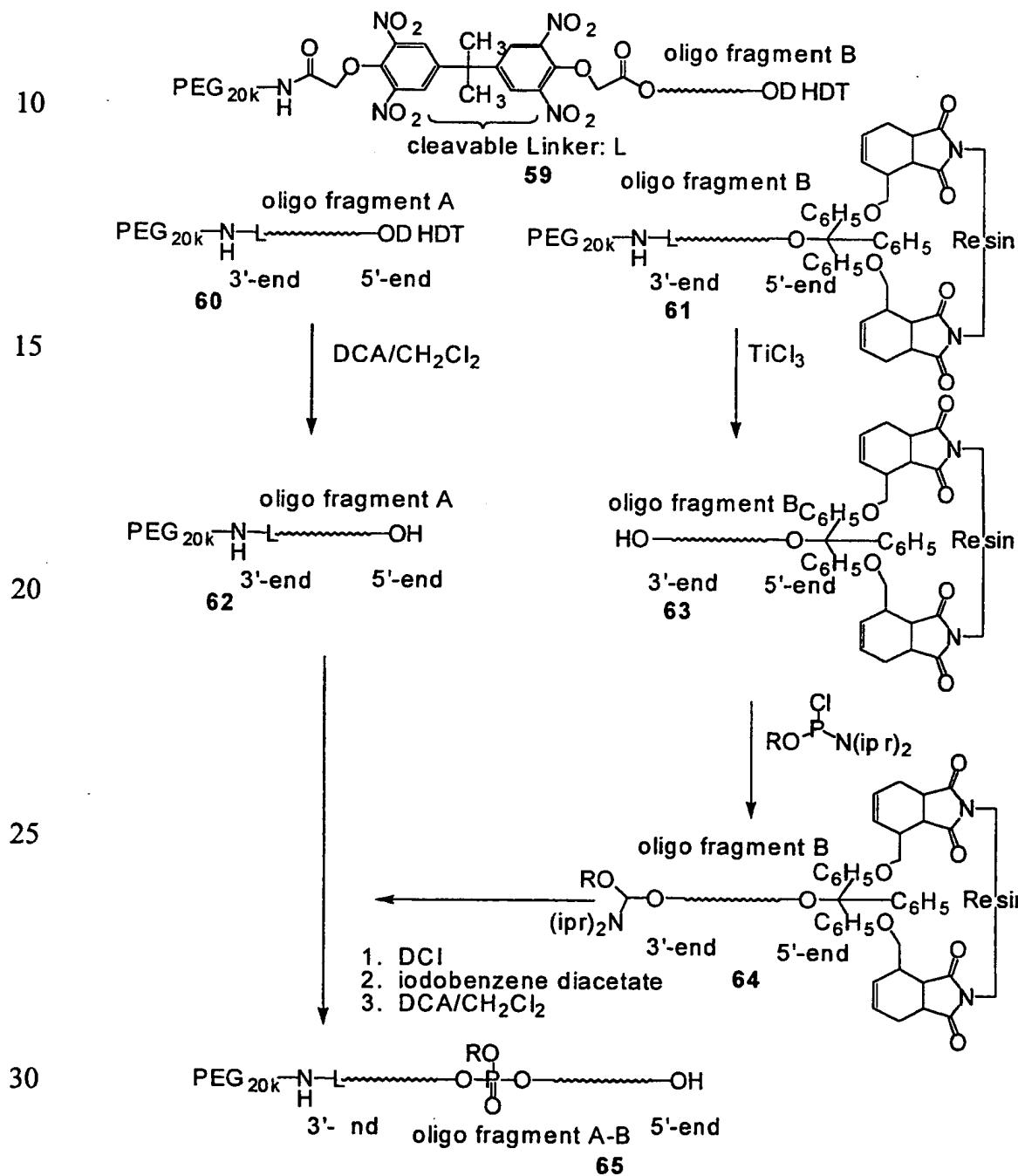
**Release/Detritylation:** 286 mg of PS-M derivatized with 11  $\mu$ mol of the [3',3'] dimer 57 was suspended in 0.25 mL dichloromethane. To this solution was added 2.6 mL of 3% DCA in dichloromethane. The PS-M immediately turned bright orange. The suspension was agitated for 5 minutes, whereupon the dichloromethane solution was removed via filtration from the PS-M. The solution obtained was immediately filtered through a pad of Dowex-Cl<sup>-</sup> ion exchange resin with dichloromethane. The filtrate was then concentrated to yield 12 mg of a white, glassy solid (contains some residual solvent and aliphatic impurities). <sup>1</sup>H NMR and <sup>31</sup>P-NMR are consistent with the desired product compound 58.

**Example 13. Preparation of an oligonucleotide from two blocks using fragment anchoring by Diels-Alder cycloadditon**

The PASS oligonucleotide synthesis scheme allows facile and efficient preparation of oligonucleotide blocks, which can be coupled to each other in a modified PASS cycle as illustrated in Scheme 16. Briefly, the oligonucleotide block 59, prepared by PASS monomer addition cycles as outlined above, is reacted with a maleimide resin to give the resin anchored oligonucleotide block 61. The 3'-terminal PEG is removed from this block by reductive cleavage of linker L with titanium trichloride, yielding resin bound fragment 63, which has a free 3'-terminus. Phosphitylation of 63 with N,N-diisopropyl-2-

cynoethyl-chlorophosphine results in the 3'-terminal phosphoramidite **64**. Compound **64** is then coupled to oligonucleotide block **62**, obtained from detritylation of oligonucleotide block **60** after capture on a maleimide resin and subsequent detritylation. The coupling reaction is followed by oxidation of the phosphite triester linkage to the corresponding phosphate triester, followed by release of the product oligonucleotide from the resin with dichloroacetic acid, giving oligonucleotide fragment **60**.

SCHEME 16



**Example 14. Automation of PASS using Diels-Alder product capture for preparation of oligonucleotides**

The coupling reagents are added to reaction vessel 212 (Figure 8) and reaction is allowed to proceed as described in Example 10. Upon completion of the coupling reaction, the reaction mixture is circulated through the diene or dienophile modified resin or membrane (hereafter referred to as the support), which is contained in vessel 214, to covalently capture the oligonucleotide. The time required for the capture step can be controlled by monitoring the disappearance of the coupling product from solution either by an HPLC or in line UV assay (not shown). The support is then rinsed to elute all failure sequences not containing the diene or dienophile. Oxidation can be accomplished either after the oligonucleotide is attached to the support or in solution prior to attachment to the dienophile support. The oxidation solution must be thoroughly removed from the resin prior to detritylation. This removal is conveniently controlled by in-line conductance monitoring (not shown). The support is then rinsed with DCA/CH<sub>2</sub>Cl<sub>2</sub> to remove the growing oligomer and captured excess monomer from the resin, thus allowing the only species in the solution to be the 5'-deprotected oligonucleotide and monomer in a DCA/CH<sub>2</sub>Cl<sub>2</sub> mixture. This mixture is then brought into contact with a membrane separator (218) to remove the DCA and the excess monomer, in addition to a solvent exchange to acetonitrile. Alternatively, the monomer may be separated by precipitation or extraction. The only species remaining in solution is the macromolecule attached oligomer in acetonitrile. This solution is now ready for the next coupling reaction.

The removal of all n-1 species with use of the dienophile support, thus eliminates the use of a capping step, and the solution is ready to be oxidized and or circulated through the dienophile support. The dienophile support can contain a cleavable linker between the dienophile moiety and the resin or membrane, such as an amide bond. This cleavable linker allows facile regeneration of the dienophile support. Linkers such as these are well known to those skilled in the art.

**Membrane Evaluation**

Recovery of Pegylated Deoxythymidine after Exposure to a Polypropylene Ultrafiltration

30 Membrane: Acetonitrile Solvent System. A solution of 2.74 mM 20k PEG-deoxythymidine (PEG-dT) was made by dissolving 1.49 grams of 46  $\mu$ mol dT/gram PEG-dT in 25 ml of

acetonitrile. Aliquots (2 mL) of the solution were then exposed to areas of 5.73 square centimeters of the working surface of a polypropylene ultrafiltration membrane (3M<sup>®</sup>) for periods of 0.25, 1 and 4 hours in 50 mL Falcon<sup>®</sup> tubes. The starting solution was rinsed from the Falcon<sup>®</sup> tubes and membranes with two 25 mL washes of acetonitrile. The wash solvent was assayed for PEG-dT spectrophotometrically by absorbance at 260 nm and balanced relative to the absorbance of the starting PEG-dT. A control to measure losses to the tube and glassware was performed by exposing a Falcon<sup>®</sup> tube without a membrane to 2 mL of the starting solution for 4 hours and assaying for PEG-dT at 260 nm. Results are shown in Table 6.

5 10 Recovery of Pegylated Deoxythymidine after Exposure to a Polypropylene Ultrafiltration Membrane: Methylene Chloride Solvent System. A solution of 2.72 mM 20k PEG-deoxythymidine (PEG-dT) was made by dissolving 1.48 grams of 46  $\mu$ mol dT/gram PEG-dT in 25 mL of methylene chloride. Aliquots (2 mL) of the solution were then exposed to areas of 5.73 square centimeters of the working surface of a polypropylene ultrafiltration membrane (3M<sup>®</sup>) for periods of 0.25, 1 and 4 hours in 50 mL Falcon<sup>®</sup> tubes. The starting solution was rinsed from the Falcon<sup>®</sup> tubes and membranes with two 25 mL washes of methylene chloride. The wash solvent was assayed for PEG-dT spectrophotometrically by absorbance at 260 nm and balanced relative to the absorbance of the starting PEG-dT. A control to measure losses to the tube and glassware was performed by exposing a Falcon<sup>®</sup> tube without a membrane to 2 mL of the starting solution for 4 hours and assaying for PEG-dT at 260 nm. Results are shown in Table 7.

15 20 Recovery of Pegylated Deoxythymidine after Exposure to a Regenerated Cellulose Ultrafiltration Membrane: Acetonitrile Solvent System. A solution of 2.85 mM 20k PEG-deoxythymidine (PEG-dT) was made by dissolving 1.55 grams of 46  $\mu$ mol dT/gram PEG-dT in 25 mL of acetonitrile. Aliquots (2 mL) of solution were exposed to areas of 5.73 square centimeters of the working surface of a polypropylene ultrafiltration membrane (Millipore<sup>a</sup>, 10KPLGC) for periods of 0.25, 1, 4 and 24 hours in 50 mL Falcon<sup>®</sup> tubes. The starting solution was rinsed from the Falcon<sup>®</sup> tubes and membranes with a 25 mL wash of acetonitrile. The membrane was soaked in 25 mL of acetonitrile for six days and then 25 30 washed with an additional 25 mL of acetonitrile. The wash solvents were assayed for PEG-dT spectrophotometrically by absorbance at 260 nm and balanced relative to the absorbance

of the starting PEG-dT. A control to measure losses to the tube and glassware was performed by exposing a Falcon® tube without a membrane to 2 mL of the starting solution for 4 hours and assaying for PEG-dT at 260 nm. Results are shown in Table 8.

Centrifugation of PEGylated Deoxythymidine in Acetonitrile / Ether: Diethyl Ether,

5 Diisopropyl Ether and N-Butyl Ethers Compared. A solution of 2.34 mM 20k PEG-deoxythymidine (PEG-dT) was made by dissolving 0.4855 grams of 46 mol/gram PEG-dT in 10 ml of acetonitrile. Aliquots of 0.5, 0.25 and 0.125 mL were precipitated by addition of 1 mL of either diethyl ether, diisopropyl ether or N-butyl ether. The precipitates were centrifuged at approximately 4,400 times gravity for 2 minutes. The PEG-dT content of the 10 supernatants was determined spectrophotometrically and balanced relative to the starting-PEG-dT. A control to show losses to handling was performed by centrifuging and assaying 1.5 mL of the starting solution by the above method. The results are summarized in Figure 9.

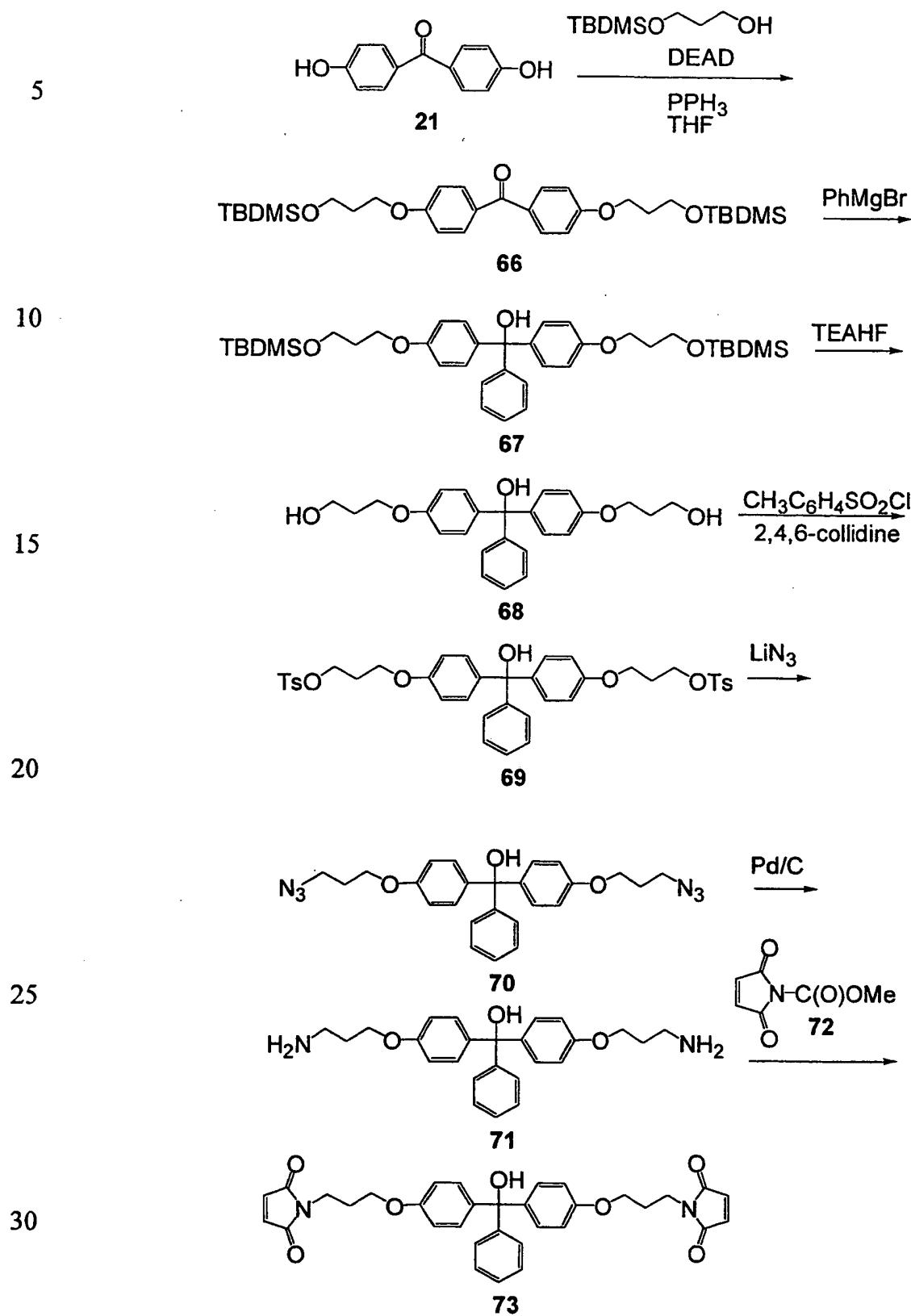
Compatibility by Flux and FTIR evaluations. Polyvinylidenedifluoride (PVDF) and

15 polypropylene membranes were evaluated by soaking them in the following solvent systems: acetonitrile, methylene chloride, the Coupling/Capping/Oxidation (c/c/0) solution in acetonitrile, and the mixture of 3% DCA in methylene chloride. Pieces of the membranes 1½" in diameter were submersed in the solutions, allowed to soak for 24 hours, placed into a membrane holder for flux evaluation of the initial solution, and then rinsed with acetonitrile 20 for further acetonitrile flux evaluations. Thus, the membrane sample was rinsed of any excess reagent that may have remained on the membrane after soaking in solution. The acetonitrile flux rates after exposure to the various solvents are listed in Table 9. As can be seen, there are only minor changes in the flux rate in (mL/min/cm<sup>2</sup>) between the PVDF and the polypropylene membranes.

25 In a retention study, the regenerated cellulose membrane was determined to have retained some of the PEG, as measured by FTIR. The silicone, ceramic, polyolefin and HDPE membranes are under investigation.

Example 15. Synthesis of maleimido-trityl monomers

SCHEME 17



## Preparation of 4,4'-di-(3-t-butylidimethylsilyloxypropoxy)-benzophenone

**(66).** 4, 4'-dihydroxybenzophenone (**21**) (10 g, 46.7 mmol) was reacted under Mitsunobu conditions with t-butyldimethylsilyloxy-3-propanol (40 g crude, approx. 150 mmol), DEAD (22.1 mL, 140.0 mmol) and triphenylphosphine (36.7 g, 140.0 mmol) in dry tetrahydrofuran 5 at 0°C. The reaction was allowed to warm to room temperature under argon. After 24 hours the reaction was concentrated and the salts precipitated with hexane/ether and filtered. The remaining material was purified by column chromatography column chromatography (silica gel; gradient of hexane to 85% hexane/ethyl acetate) to afford to afford 14 g of the desired product compound **66** in 54% yield.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.92 (d, 4H), 10 7.68 (d, 4H), 4.13 (t, 4H), 3.76 (t, 4H), 1.96-1.88 (m, 4H), 0.85 (s, 18H), 0.02 (s, 12H).

## Preparation of 4,4'-di-(3-t-butyldimethylsilyloxypropoxy)-triphenylmethanol

**(67).** The protected benzophenone **66** (5.7 g, 10.2 mmol) was dissolved in 40 mL dry THF and phenylmagnesium bromide (20.5 mL, 20.4 mmol) was added. The reaction was stirred under argon at room temperature for 2 hours, concentrated, partitioned between dichloromethane and saturated ammonium chloride, and washed with water. The organic layer was dried ( $\text{MgSO}_4$ ) and concentrated to yield 6.5 g of a yellow gum, compound **67**, in quantitative yield and used directly in the next step.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.27-7.17, 7.05, 6.82 (m, 13H), 6.23 (s, 1H), 3.99 (t, 4H), 3.73 (t, 4H), 1.91-1.83 (m, 4H), 0.84 (s, 18H), 0.02 (s, 12H).

20 Preparation of 4,4'-di-(3-hydroxypropoxy)-triphenylmethanol (68). The trityl compound **67** (6.37 g, 10 mmol) was deprotected by treatment with triethylamine hydrofluoride (3.64 g, 30 mmol) in acetonitrile at room temperature for 16 hours. The reaction was concentrated and purified by column chromatography (silica, gradient: 1:1 hexane:ethyl acetate to ethyl acetate:5% methanol all with 1% triethylamine) affording 2.8 g  
25 of the desired product **68** as a yellow gum in 69% yield.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  7.30-7.18, 7.06, 6.83 (m, 13H), 6.22 (s, 1H), 4.55 (t, 2H), 4.07-3.98 (m, 4H), 3.57-3.52 (m, 4H), 1.88-1.80 (m, 4H).

## Preparation of 4,4'-di-(3-p-toluenesulfonyloxypropoxy)-triphenylmethanol

(69). A solution of tosyl chloride (1.43 g, 7.49 mmol) and 2,4,6-collidine (1 mL, 7.49 mmol) in acetonitrile was added to compound 68 (1.39 g, 3.4 mmol) in 15 mL acetonitrile. The reaction was stirred at room temperature under argon for 2.5 days and then

concentrated. The residue was purified by column chromatography (silica, 60% ethyl acetate in hexane with 1% triethylamine) to give 0.6 g of the tosylated compound **69** in 25% yield. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.76 (d, 4H), 7.38 (d, 4H), 7.32-7.06, 7.01, 6.73 (m, 13H), 6.26 (s, 1H), 4.17 (t, 4H), 3.88 (t, 4H), 2.33 (s, 6H), 2.04-1.96 (m, 4H).

5        Preparation of 4,4'-i-(3-azidopropoxy)-triphenylmethanol (70). To a solution of **69** (0.6 g, 0.84 mmol) in 15 mL of dry DMF was added lithium azide (0.12 g, 2.51 mmol). The reaction was stirred under argon at room temperature overnight, concentrated and purified by column chromatography (silica, 60% ethyl acetate in hexane with 1% triethylamine) to yield 0.38 g (100 %) of compound **70** as a yellow gum. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.34-7.17, 7.07, 6.85 (m, 13H), 6.25 (s, 1H), 3.99 (t, 4H), 3.50 (t, 4H), 2.00-1.77 (m, 4H).

10        Preparation of 4,4'-di-(3-aminopropoxy)-triphenylmethanol (71). The azide (**70**) (0.25 g, 0.55 mmol) was warmed with activated charcoal in methanol, filtered and concentrated. The residue was again dissolved in 50 mL of methanol and 55 mg 5% 15 palladium on carbon was added. The flask was evacuated and a hydrogen filled balloon added. After 1 hour at room temperature the catalyst was filtered. The reaction was concentrated and used directly in the next step.

15        Preparation of 4,4'-di-(3-maleimidopropoxy)-triphenylmethanol (73). The crude residue **71** was dissolved in 50 mL 1:1 acetonitrile:water and stirred in an ice bath. 20 Methoxy carbonyl maleimide reagent (**72**) (0.16 g, 0.98 mmol) was added and over 2 hours the pH was observed to drop from 10.1 to 5. The pH was then adjusted to 2 with 1 M sulfuric acid and the reaction concentrated. The residue was partitioned between ethyl acetate and brine. The organic layer was concentrated, re-dissolved in 1:1 acetonitrile:water and stirred with 10 mL 5% sodium bicarbonate. After 17 minutes the reaction was acidified 25 to pH 3 with 1 M sulfuric acid. Ethyl acetate (20 mL) was added and the solution was partitioned and the aqueous layer back extracted with ethyl acetate. The combined organic layers were concentrated and purified by column chromatography (silica, ethyl acetate and hexane mixtures) to give 0.104 g of product **73** in 36% yield. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 7.30-7.18, 7.04, 7.68 (m, 13H), 7.02 (s, 4H), 6.24 (s, 1H), 3.92 (t, 4H), 3.57 (t, 4H), 1.99-30 1.89 (m, 4H). MS (MS+566). Anal. Calcd. for C<sub>33</sub>H<sub>30</sub>N<sub>2</sub>O<sub>7</sub>: C, 69.95; H, 5.34; N, 4.94. Found: C, 69.74; H, 5.67; N, 4.78.

Example 16. Selective removal of failure sequences during non-PASS oligonucleotide synthesis by capping with a diene-modified capping reagent and subsequent capture of such species on a dienophile resin or membrane

Preparation of 3,5-hexadienoic acid anhydride (74), 3,5-hexadienoxyacetic anhydride (75) and trihexadienoysilyl chloride (76). Compounds 74, 75 and 76 (Scheme 18) are prepared by standard methods known in the field. Compound 74 can be prepared from the 3,5-hexadienol by oxidation to the corresponding hexadienoic acid and subsequent dehydration. Compound 75 is obtained from reaction of iodoacetic anhydride with 3,5-hexadienol and compound 76 is a product of the reaction of silicon tetrachloride with 3,5-hexadienol. In addition to these methods of synthesis, compounds 74, 75 and 76 can be prepared by a variety of other methods.

Use of compound 75 as capping reagent and subsequent failure removal during 3'-PEG anchored solution phase synthesis. A solution phase synthesis is performed as described in Example 7 with the exception that the capping reagent is altered and that a failure subtraction step is added. During the capping step equal amounts of 3,5-hexadienoxyacetic anhydride (75), 2,6-lutidine, and N-methylimidazole are simultaneously injected into the solution and stirred. Maleimide-derivatized polystyrene resin is added to the reaction mixture and stirring is continued. The resin is filtered off and the polymer is precipitated from ether as described in the detritylation procedure of Example 7.

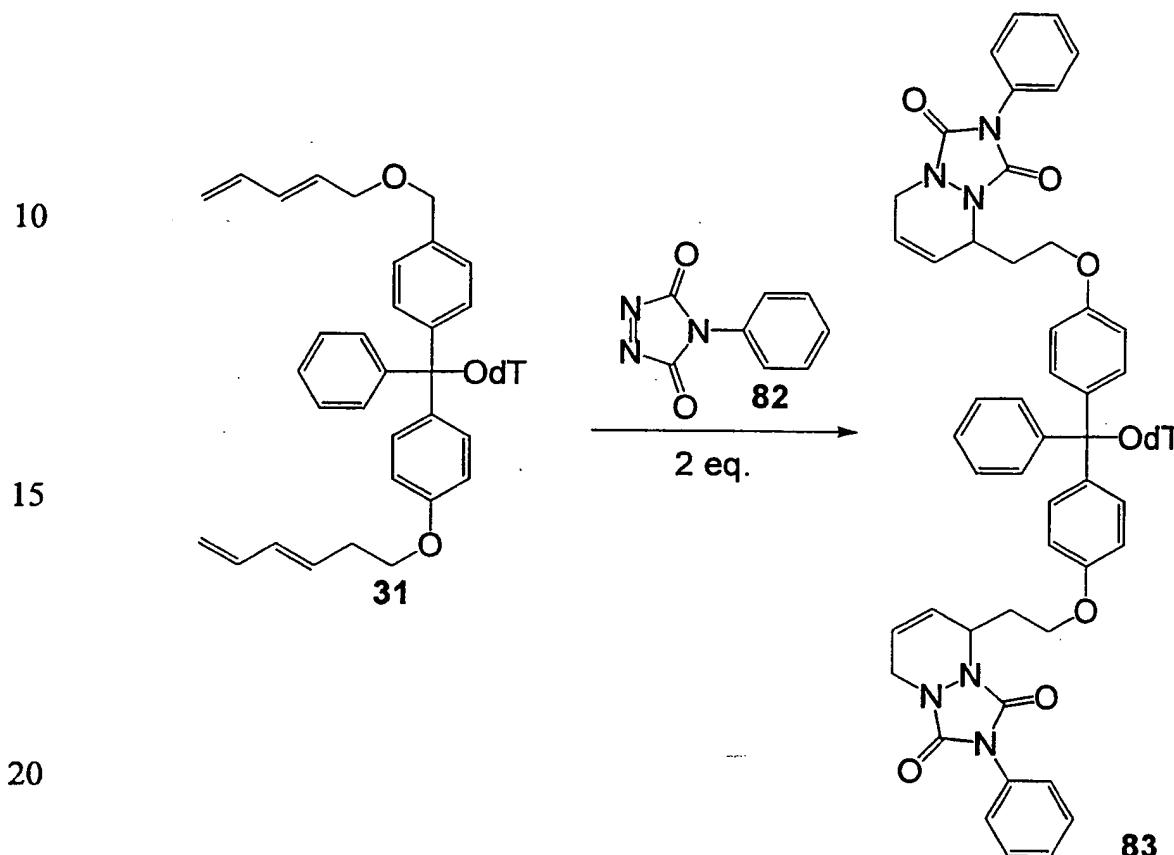
Use of compound 76 as capping reagent and failure removal during conventional solid phase synthesis. Conventional solid phase synthesis of DNA, RNA, and modified oligonucleotides is carried out according to the specifications given by the solid phase synthesizer manufacturer with the exception that tri(3,5-hexadienoxy)silyl chloride 76 is substituted for acetic anhydride in the capping reagent. Upon cleavage and deprotection of the oligonucleotide from the support the crude oligonucleotide is taken up in water/acetonitrile and maleimide-derivatized polystyrene is added to the solution. Upon complete reaction, the resin-bound failure sequences are filtered off and the product oligonucleotide is further purified if required.

Example 17. Diels-Alder Reaction of 5'-DHDTO-dT with PTAD

Scheme 22 describes the Diels-Alder reaction of the diene substituted trityl alcohol 5'-O-(4,4'-di-3,5-hexadienoxytrityl)thymidine (5'-DHDTO-dT) (31) with PTDA (82).

5

SCHEME 22



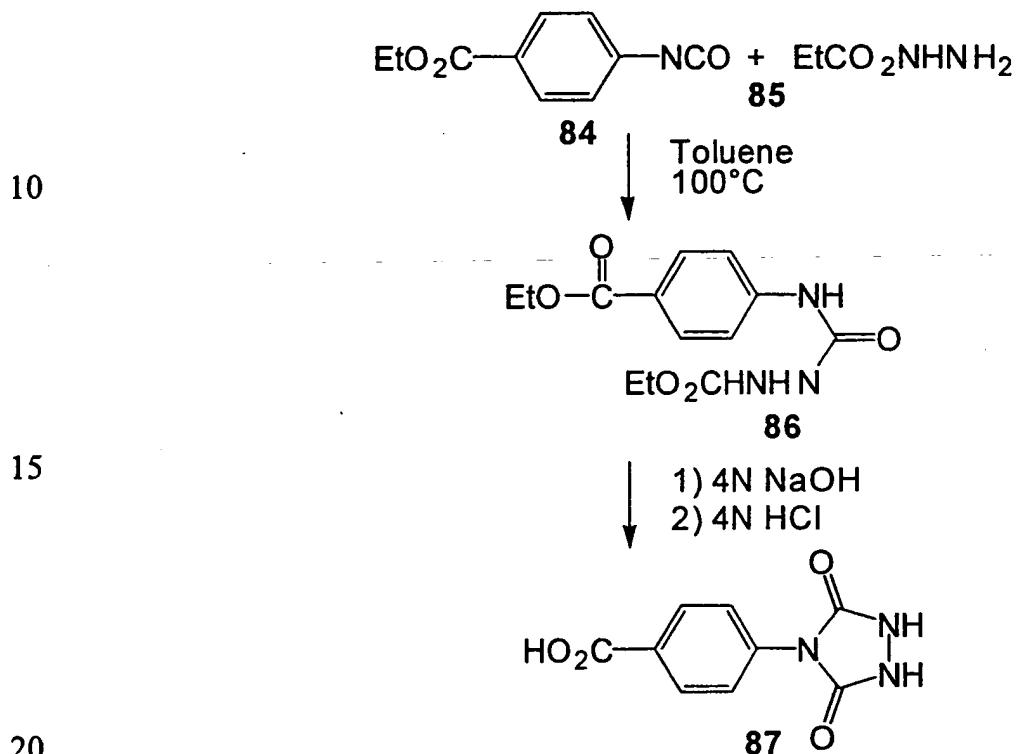
To a solution of 51 mg (31  $\mu$ mol) 5'-DHDTO-dT (31) in 3 mL of anhydrous  $\text{CDCl}_3$  was added a solution of 22 mg (120  $\mu$ mol) of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (82) in 1.2 mL of  $\text{CDCl}_3$  (0.1 M) in 0.1 mL aliquots under an atmosphere of Ar. The deep red color of the PTAD bleached almost immediately upon addition to the 5'-DHDTO-dT (31) solution. The reaction was monitored by  $^1\text{H}$  NMR. Comparison of the area under a peak unique to the starting material (31), to that of a peak unique to the Diels-Alder adduct yields the graph depicted in Figure 10 in which complete reaction, as represented by the disappearance of the peak due to 5'-DHDTO-dT (31) at 5.80 ppm, is

shown to occur upon the addition of 0.6 mL (60  $\mu$ mol) of the 0.1 M PTAD solution to form the bis-Diels Alder adduct **83**, as expected.

Example 18. Preparation of 4-(4-urazole)benzoic acid (87)

5 Scheme 23 illustrates the synthesis of 4-(4-urazole)benzoic acid (87).

SCHEME 23



10 Preparation of semicarbazide (86). To a solution of 10 g (52 mmol) of ethyl 4-isocyanatobenzoate (**84**) in 100 mL of toluene was added 54 g of ethyl carbazole (**85**). The reaction mixture was refluxed for 2 hours, during which time a white precipitate formed. The reaction mixture was cooled in an ice bath for 10 minutes, after which the 15 solids were filtered and washed with 2x100 mL toluene. After drying under vacuum for 14 hours, 14.8 g of a white solid, semicarbazide **86** was isolated (96% yield).  $^1$ H NMR (300 MHz,  $d_6$ -DMSO)  $\delta$  9.18 (s, 1H), 9.00 (s, 1H), 8.40 (s, 1H), 7.95 (d,  $J$  = 9.5 Hz, 2H), 7.74 (d,  $J$  = 9.5 Hz, 2H), 4.26 (q,  $J$  = 7.8 Hz, 2H), 4.06 (q,  $J$  = 7.5 Hz, 2H), 1.29 (t,  $J$  = 7.8 Hz, 3H), 20 1.22 (t,  $J$  = 7.5 Hz, 3H).  $^{13}$ C NMR (300 MHz,  $d_6$ -DMSO)  $\delta$  165.5, 156.9, 144.4, 130.2, 122.7, 117.6, 60.6, 60.3, 14.5, 14.2.

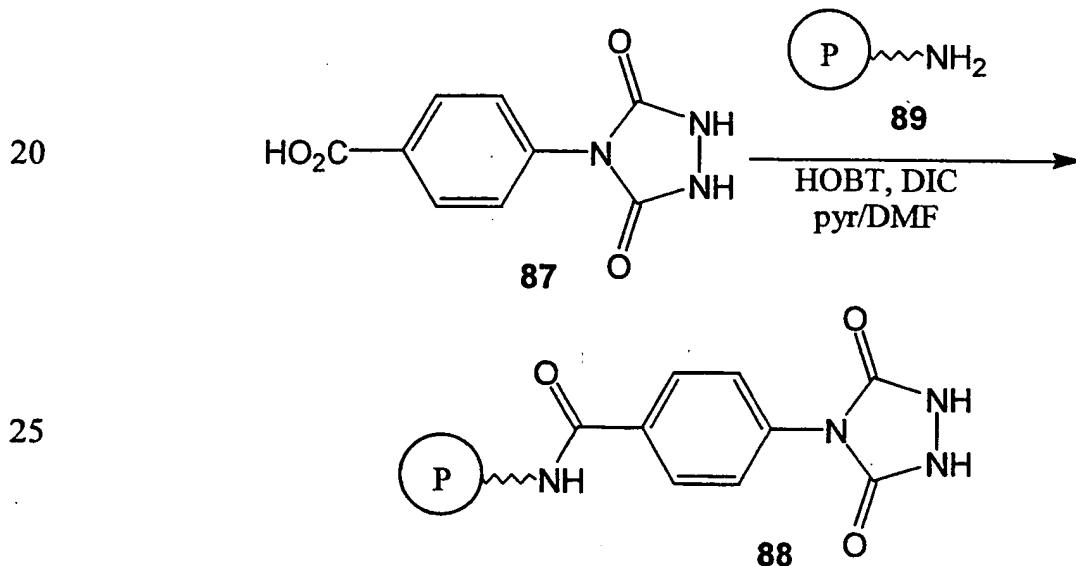
Preparation of 4-(4-urazole)benzoic acid (87). Semicarbazide **86** (14.6 g, 49 mmol) was suspended in 100 mL of 4 N NaOH and refluxed for 2 hours, during which time the white solid gradually dissolved. After cooling in an ice bath for 25 minutes the pH of the reaction mixture was adjusted to 1 via dropwise addition of 4 N HCl and a white solid 5 precipitated from solution. The solid was filtered, washed with 4x300 mL of water and dried under vacuum, over  $P_2O_5$ , for 18 hours to yield 11.0 g (100% yield) of compound **(87)**.  $^1H$  NMR (300 MHz,  $d_6$ -DMSO)  $\delta$  13.09 (br. s, 1H), 10.68 (br. s, 2H), 8.31 (d,  $J$  = 9.5 Hz, 2H), 7.66 (d,  $J$  = 9.5 Hz, 2H).  $^{13}C$  NMR (300 MHz,  $d_6$ -DMSO)  $\delta$  166.8, 152.8, 136.0, 129.9, 129.5, 125.3.

10

**Example 19. Derivatization of amino-functionalized resins with 4-(4-urazole)benzoic acid**

Scheme 24 illustrates a general procedure for the derivatization of amino-functionalized resins with 4-(4-urazole)benzoic acid **(87)**. This general procedure has been successfully applied to a variety of amino-functionalized resins, including aminopropyl 15 silica gel, aminopropyl CPG, NovaSyn<sup>®</sup> TG amino resin HL, and ArgoGel<sup>TM</sup> amino resin.

SCHEME 24



Preparation of urazol derivatized resin (88).

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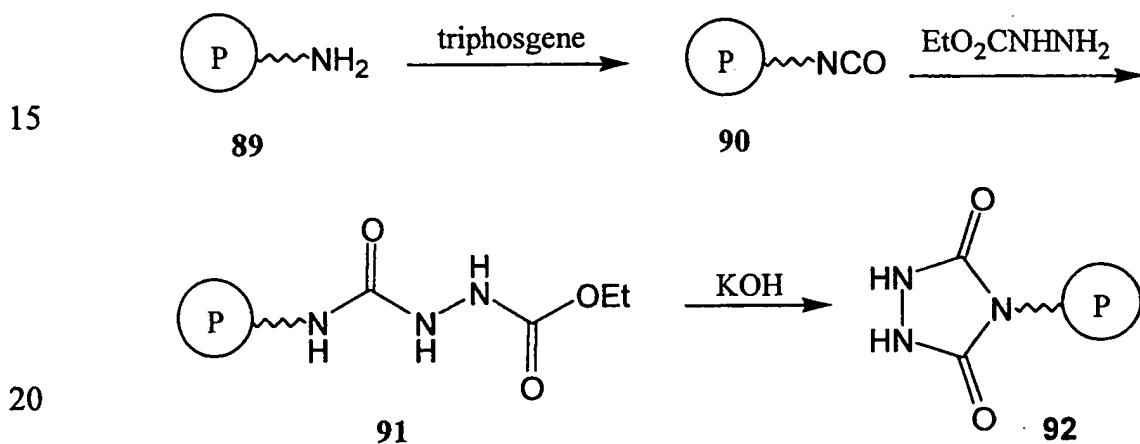
A suspension of 10.1 g of aminomethyl polystyrene (loading = 0.9 mmol/g), preswollen in 50 mL DMF, was added to a solution of 3.13 g (14 mmol) of urazole **87** and

2.3 g (16.9 mmol) HOBT in 100 mL DMF containing 5% pyridine. To this reaction mixture was added 7.3 mL (47 mmol) DIC. The reaction was gently agitated for 24 hours, the solid filtered and then washed with 2x100 mL DMF, 2x100 mL MeOH, 2x100 mL  $\text{CH}_2\text{Cl}_2$ , 2x100 mL acetone and finally 2x100 mL  $\text{CH}_2\text{Cl}_2$ . After drying under vacuum, over  $\text{P}_2\text{O}_5$  for 5 48 hours, the solids tested negative for free primary amino functionalities by ninhydrin titration.

Example 20. Derivatization of amino-functionalized resins via direct formation of a urazole on resin

10 Scheme 25 illustrates a general method for the derivatization of amino-functionalized resins via direct formation of the urazole on the resin.

SCHEME 25



Preparation of isocyanate-derivatized TG amino resin (90). A suspension of 5.4 g of TentaGel- $\text{NH}_2$  resin 89 was preswollen in 40 mL  $\text{CH}_2\text{Cl}_2$ . TEA (0.5 mL) was added to this suspension and the mixture was agitated for 5 minutes. Triphosgene (186 mg) was 25 then added and the reaction mixture was agitated for an additional 3 hours at room temperature, after which the reaction mixture was filtered and the solids washed with 3x100 mL  $\text{CH}_2\text{Cl}_2$  to yield 5.3 g of a yellow solid (90). Titration with ninhydrin gave a negative test for primary amine groups (although allowing the material to sit in the ninhydrin/acid solution resulted in a very slow formation of the dark blue color indicative of the free 30 amines, due to degradation of the isocyanate). An IR spectrum obtained of the solid by diffuse reflectance methods shows a strong isocyanate peak at  $2285 \text{ cm}^{-1}$ .

Preparation of urazole-derivatized TG amino resin (92). To a suspension of 5.13 g of TG-isocyanate resin **90** in 35 mL toluene was added 186 mg ethyl carbazole and the reaction mixture was heated to 85°C. After 2.5 hours the reaction mixture was filtered and the solids were washed with 3x50 mL toluene and 3x50 mL CH<sub>2</sub>Cl<sub>2</sub> to yield 5.4 g of 5 TG-semicarbazate resin **91**. The IR spectrum showed no remaining isocyanate. A definitive set of carbonyl absorbances appear at 1742 and 1701 cm<sup>-1</sup>. The solids were resuspended in 20 mL of 2 N KOH and heated to 95°C for 45 minutes. The reaction mixture was then 10 filtered and the solids washed with 3x40 mL water, 3x40 mL MeOH and 3x40 mL CH<sub>2</sub>Cl<sub>2</sub>. The IR spectrum of the product, urazole derivatized resin **92**, which has a single carbonyl peak at 1696 cm<sup>-1</sup> is distinctly different from the starting TG resin **90**.

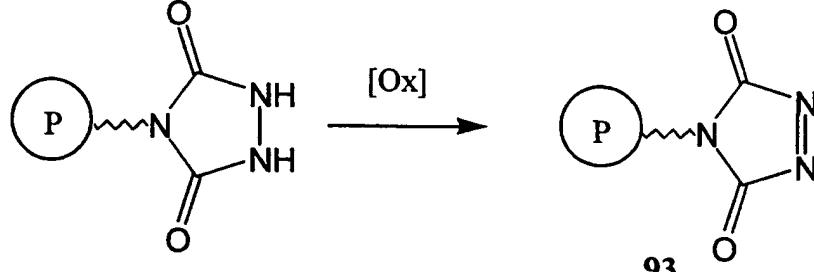
**Example 21. Oxidation of a urazole functionalized solid support to the 1,2,4-triazoline-3,5-dione (93)**

Example 20 describes a variety of methods for oxidizing a urazole 15 functionalized solid support to the 1,2,4-triazoline-3,5-dione (TAD) (Scheme 26). Oxidation of the PTAD is sensitive to the presence of nucleophiles. Oxidation of the urazole to TAD gives a bright red, orange or purple solution as described in the literature. (Keana *et al.* (1983) *Org. Chem.* **48**:1982).

SCHEME 26

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Oxidation with pentafluoroiodobenzene bis(trifluoroacetate). Oxidation of

both soluble urazole **87**, as well as a number of urazole-derivatized solids, proceeded as expected in both DMF and CH<sub>2</sub>Cl<sub>2</sub> with pentafluoro-iodobenzene bis(trifluoroacetate).

Compatibility of this oxidant with a DHDT-protected oligonucleotide was tested by

30 performing the Diels-Alder reaction in the presence of the oxidant. Therefore, a solution of 3'-HO-dT-5'-ODHDT in DMF was added to the oxidized resins in the presence of the

oxidizing agent. With DMF as the solvent, 3'-HO-dT-5'-ODHDT effectively bleached red color from the solid supports, which has been shown to be a reliable indication that the Diels-Alder reaction is taking place. However, with  $\text{CH}_2\text{Cl}_2$  as the solvent, detritylation of the DHDT moiety occurs rapidly upon addition of the 3'-HO-dT-5'-ODHDT to the oxidized 5 TAD-resins.

Oxidation with dinitrogen tetroxide. One of the more widely used reagents for oxidizing urazoles to 1,2,4-triazoline-3,5-diones is dinitrogen tetroxide ( $\text{N}_2\text{O}_4$ ). This oxidant proved effective in oxidizing the soluble urazole 87, as well as the TG amino resin which had been directly derivatized through the isocyanate to the urazole (see Example 20).

10 This oxidant is not effective in oxidizing urazole bound to resins via an amide linker.

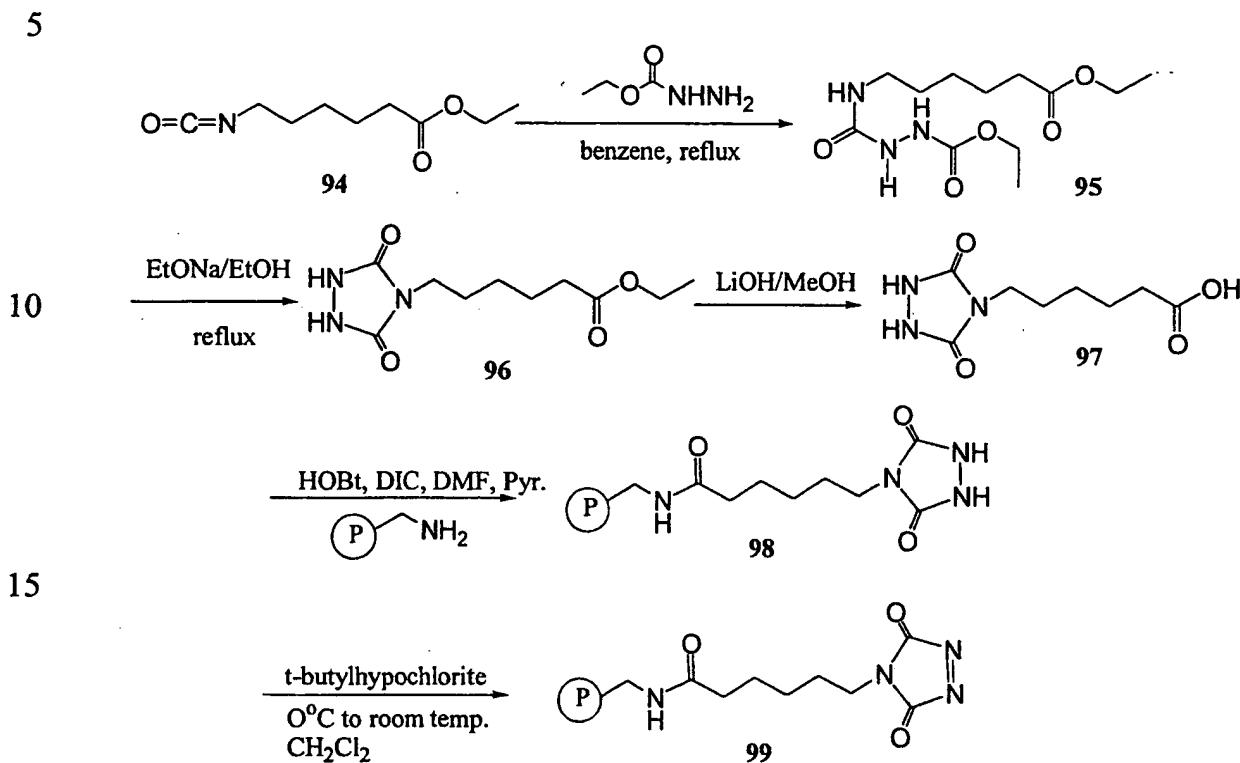
Oxidation with N-bromosuccinimide. N-bromosuccinimide (NBS) is a stable, relatively safely handled oxidant that forms TADs from the urazole precursors. It is freely soluble in DMF and relatively soluble in  $\text{CH}_2\text{Cl}_2$ . This oxidant has proven effective in oxidizing the soluble urazole 87, as well as oxidizing urazole bound to a variety of resins 15 via an amide linker (Example 19). This oxidant, however, is not effective in oxidizing the TG amino resin which had been directly derivatized through the isocyanate to the urazole (Example 20). Thus, this oxidant has complimentary reactivity to  $\text{N}_2\text{O}_4$  (see above). The primary drawback to using NBS or  $\text{N}_2\text{O}_4$  as oxidizing agents, is that both oxidants release acidic byproducts upon oxidation of the urazole substrate, which result in rapid detritylation 20 of the DHDT moiety if not removed from the solid supports prior to introduction of the DHDT-protected oligonucleotide.

General procedure for oxidation of urazole-derivatized solid supports with NBS. A solution of 130 mg (0.73 mmol) N-bromosuccinimide in 0.73 mL DMF (0.1 M) was added to a suspension of 0.3 g (loading = 0.9 mmol/g) polystyrene-urazole which had 25 been preswollen in 1.5 mL  $\text{CH}_2\text{Cl}_2$ . The solids turned reddish-orange and the solution turned bright yellow immediately upon addition. The oxidation was allowed to proceed for 30 to 45 minutes, at which time the solids were filtered and washed with 4x15 mL DMF (until the DMF filtrate was clear and colorless) followed by 4x20 mL  $\text{CH}_2\text{Cl}_2$ . The solids turned bright red upon replacement of the DMF solvent with  $\text{CH}_2\text{Cl}_2$ . The oxidized material 30 was used immediately, although the polystyrene-TAD has tentatively been observed to be stable upon removal of all solvent and storage.

Example 22. Derivatization of aminomethylpolystyrene resin with urazole caproic acid

Scheme 27 illustrates the synthesis and derivatization of aminomethylated polystyrene resin with urazole caproic acid.

SCHEME 27



**Preparation of the semicarbazide of ethyl 6-isocyanatohexanoate (95).** A solution of ethyl 6-isocyanatohexanoate (94) (8.0 g, 43.2 mmol, commercially available from Lancaster) in benzene (108 mL) was treated with ethyl carbazole (4.95 g, 47.51 mmol) and the mixture was heated to reflux for 1 hour under an argon atmosphere. The solution was allowed to gradually cool to room temperature. The reaction mixture was concentrated *in vacuo* to obtain a white solid which was dissolved in hot ethyl acetate (170 mL). Upon addition of hot hexane (260 mL) a white precipitate formed. The solid, semicarbazide 95, was collected (11.22 g, 90% yield) and characterized by <sup>1</sup>H NMR, <sup>13</sup>C NMR, IR, MS, and elemental analysis. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 6.69 (s, 1H), 4.21 (q, J = 6.90 Hz, 2H), 4.13 (q, J = 7.23 Hz, 2H), 3.24 (t, J = 6.93 Hz, 2H), 2.30 (t, J = 7.23 Hz, 2H), 1.64 (m, 2H), 1.53 (m, 2H), 1.35 (m, 2H), 1.29 (t, J = 6.93 Hz, 3H), 1.26 (t, J = 7.23 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) δ 173.69, 158.97, 157.51, 62.03, 60.18, 39.68, 34.04, 29.53, 26.11, 24.42,

14.30, 14.10. IR (CDCl<sub>3</sub>) 3344, 2976, 2928, 1722, 1686, 1544, 1235 cm<sup>-1</sup>. MS *m/e* for C<sub>12</sub>H<sub>23</sub>N<sub>3</sub>O<sub>5</sub>: calcd 289.16; found 288.2. Anal. calcd for C<sub>21</sub>H<sub>23</sub>N<sub>3</sub>O<sub>5</sub>: C, 49.81; H, 8.01; N, 14.52. Found: C, 49.92; H, 8.17; N, 14.63.

Preparation of 6-(4-urazole)caproic acid ethyl ester (96). A solution of 5 semicarbazide **95** (11.31 g, 39.09 mmol) in absolute ethanol (250 mL) was treated with sodium ethoxide (5.32 g, 78.18 mmol) under an argon atmosphere. The yellow-orange solution was heated to reflux for 15 hours during which time the solution became pale yellow. The solution was allowed to gradually cool to room temperature and the pH was adjusted to 3 using 1 M HCl/EtOH. The solution was then filtered and the filtrate was 10 concentrated *in vacuo*. The residue was taken up in CH<sub>2</sub>Cl<sub>2</sub> and concentrated. This procedure was repeated three times and the residue was allowed to stand under high vacuum overnight. The product, compound **96**, was used without further purification. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ 4.13 (t, J = 7.23 Hz, 2H), 3.55 (t, J = 6.93 Hz, 2H), 2.31 (t, J = 7.23 Hz, 2H), 1.69 (m, 4H), 1.38 (m, 2H), 1.26 (t, J = 7.23 Hz, 3H). <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>) 15 δ 177.38, 173.86, 155.70, 60.39, 38.81, 33.97, 27.49, 25.90, 24.23, 14.06. IR (CDCl<sub>3</sub>) 3166, 3048, 2942, 1725, 1672, 1478, 11.78 cm<sup>-1</sup>. MS *m/e* for C<sub>10</sub>H<sub>17</sub>N<sub>3</sub>O<sub>4</sub>, calcd 243.26; found 241.9.

Preparation of the 6-(4-urazole)caproic acid (97). A solution of compound **96** (39.09 mmol) in methanol (250 mL) and water (80 mL) was treated with LiOH·H<sub>2</sub>O and 20 the mixture was stirred for 3 hours. The methanol was removed *in vacuo* and the pH was adjusted to 2 with 1 M HCl. The aqueous solution was transferred to a separatory funnel and was extracted several times with ethyl acetate and methylene chloride. The combined organic layers were dried (Na<sub>2</sub>SO<sub>4</sub>), filtered and concentrated to obtain 6.14 g of urazole **97** (73.6% yield from **96**) as a white solid which was used without further purification. <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD) δ 3.48 (t, J = 7.23 Hz, 2H), 2.27 (t, J = 7.26 Hz, 2H), 1.63 (m, 2H), 1.35 (m, 2H). <sup>13</sup>C NMR (75 MHz, CD<sub>3</sub>OD), δ 180.17, 159.90, 42.34, 37.45, 31.49, 29.90, 28.30. IR (KBr pellet) 3188, 2944, 1753, 1725, 1698, 1674, 1476, 1350, 1227 cm<sup>-1</sup>. MS *m/e* for C<sub>8</sub>H<sub>13</sub>N<sub>3</sub>O<sub>4</sub>, calcd 215.21; found, 213.9.

Preparation of aminomethylated polystyrene resin derizatized with urazole 30 caproic acid. A suspension of aminomethylated polystyrene resin (6.11 g, loading = 1.13 mmol/gram, 6.90 mmol), preswollen in DMF (60 mL) for 40 minutes, was mixed with 6-(4-

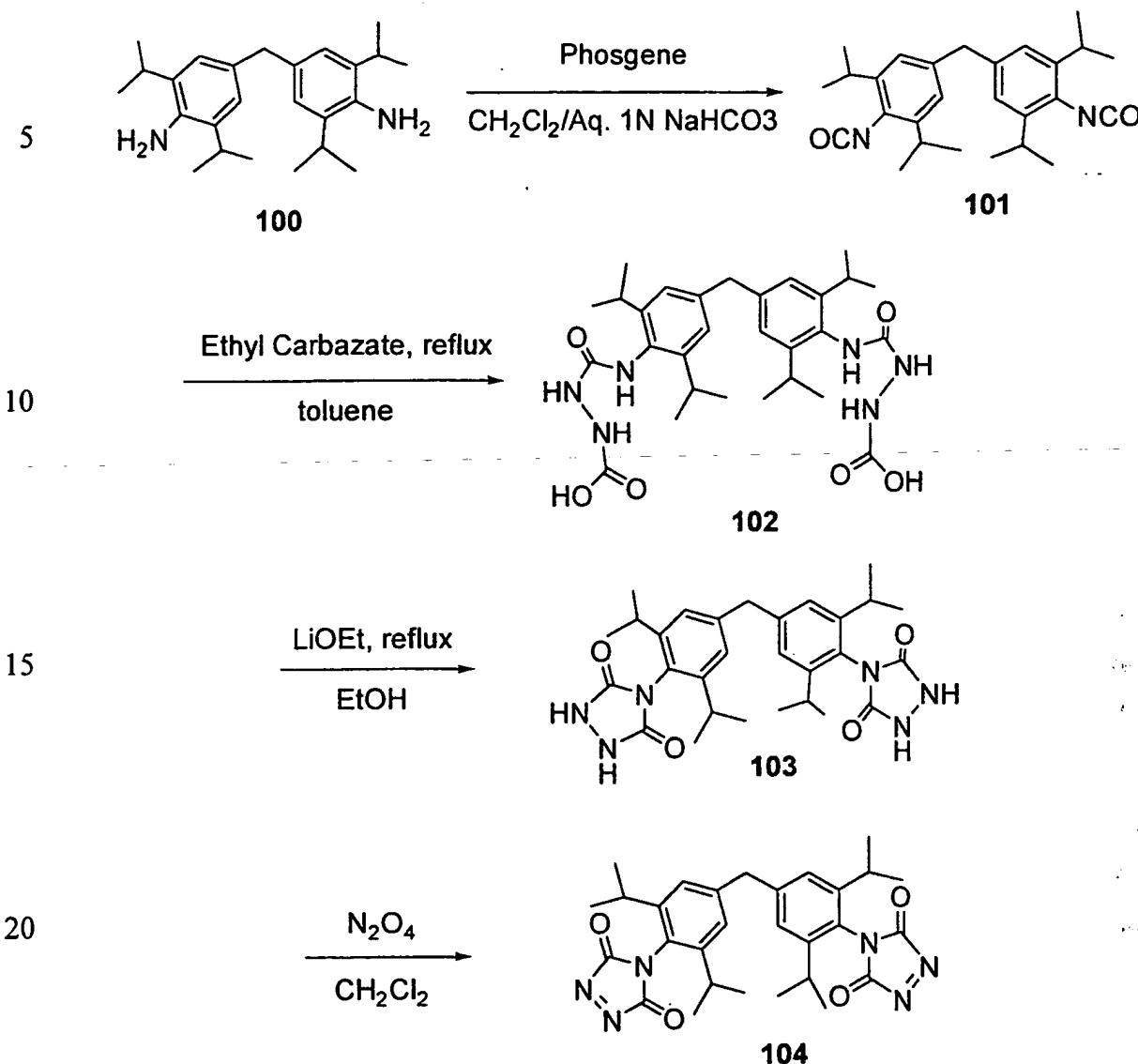
urazole)caproic acid (97) (1.60 g, 7.53 mmol), 1,3-diisopropylcarbodiimide (DIC) (4.45 g, 5.5 mL, 35.26 mmol), 1-hydroxybenzotriazole (BtOH) (1.93 g, 12.61 mmol) and pyridine (42 mL). The suspension was allowed to shake for 15 hours under argon. The solvent was then filtrated off and the resin was washed with DMF (2x50 mL), MeOH (2x50 mL), 5 methylene chloride (2x50 mL), acetone (2x50 mL) and methylene chloride (2x50mL). The resin was dried in a desiccator over  $P_2O_5$  under reduced pressure for 24 hours to yield 8.12 g of the 6-(4-urazole)caproic acid derivatized 2% aminomethylated polystyrene resin (98). The resin was analyzed by elemental analysis: calcd loading = 1.16 mmol/gram.

Preparation of TAD-caproamide resin (99). A suspension derivatized resin 10 **98** (0.520 g, manufacturing loading 1.38 mmol per g, 0.718 mmol) in methylene chloride (10 mL) was allowed to gently shake for 10 minutes under argon. The suspension was cooled to 0°C and treated with t-butyl hypochlorite (0.173 g, 1.59 mmol). The suspension instantly turned bright pink and was gently shaken for 30 minutes while it reached room 15 temperature. The solvent was drained off by filtration through a gas dispersion tube attached to a vacuum line. The resin was washed with methylene chloride (2x10 mL), DMF (2x10 mL) and methylene chloride (2x10 mL). The pH was monitored and at the end of the washes it was neutral. The resin was dried under high vacuum over  $P_2O_5$  for 15 hours to obtain 0.360 grams of a dark pink resin (99) (TAD-caproamide resin). IR (KBr) 1754, 1672 20  $cm^{-1}$ . Analysis by titration using trans, trans-1,4-diphenyl-1,3-butadiene showed loading of 0.46 mmol per gram. Oxidation and titration of a second batch of resin showed a loading of 0.78 mmol per gram.

Example 23. Preparation of dienophile derivatized resins by mono Diels-Alder reaction of a bis-TAD

25 Preparation of bis-1,2,4-triazoline-3,5-dione (bis-TAD) (104). Scheme 28 illustrates the synthesis of bis-TAD (104).

## SCHEME 28

Preparation of semicarbazide (102). 4,4'-Methylene-bis(2,6-

25 diisopropylaniline) (**100**) (30.0 g, 81.9 mmol) was weighed into a 1 L round bottom flask which was septum sealed and purged with argon. The flask was charged with 500 mL of methylene chloride with stirring. Phosgene (105 mL of a 1.93 M solution in toluene, 1.25 equiv.) was added rapidly via syringe and the mixture was stirred for 5 minutes. A solution of aqueous saturated  $\text{NaHCO}_3$  (200 mL) was added to the stirring reaction mixture and the mixture was stirred vigorously for 30 minutes. The reaction mixture was poured into a 2 L separatory funnel and the aqueous phase removed and discarded. The mixture was washed

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with additional saturated  $\text{NaHCO}_3$  (2x100 mL) and brine (1x100 mL). The organic phase was dried with  $\text{Na}_2\text{SO}_4$ , filtered and the solvent removed *in vacuo*. The resultant oil (101) was redissolved in toluene and transferred to a 1 L, 3 neck round bottom flask. Enough toluene was added to total 500 mL and the flask was purged with argon. Ethyl carbazate 5 (25.5 g, 1.5 equiv.) was added all at once with vigorous stirring for 15 minutes. The flask was fitted with a reflux condenser and the mixture was refluxed for 18 hours. The mixture was cooled to room temperature and the resultant precipitate collected, washed with methylene chloride and dried under high vacuum, to give 44.5 g of the semicarbizide 102.  $^1\text{H}$  NMR gives spectra consistent with pure product.

10 Preparation of bis-urazole (103). A three neck, 500 mL round bottom flask was purged with argon. The flask was charged with absolute ethanol (500 mL) and cooled to 0°C. Lithium hydride (2.60 g, 327 mmol) was added all at once with vigorous stirring and allowed to react for 15 minutes. The flask was brought to room temperature with stirring and an argon purge. Semicarbizide 102 (25.0 g, 40.9 mmol) was added to the flask 15 all at once and the flask was fitted with a reflux condenser. The mixture was brought to reflux and allowed to react for 18 hours. The resultant precipitate was filtered and redissolved in deionized water. Concentrated HCl was added until the pH was about 1. The precipitate was collected, coevaporated with absolute ethanol and dried under high vacuum overnight. The reaction afforded 20.6 g of the bis-urazole (103).  $^1\text{H}$  NMR gives spectra 20 consistent with pure product.

25 Preparation of bis 1,2,4-triazoline-3,5-dione (104). Bis-urazole (103) (18.0 g, 33.6 mmol) was suspended in methylene chloride (150 mL) with stirring and cooled to 0 °C. Nitrogen tetroxide was bubbled through the solution for 10 minutes from a cylinder that was fully opened. The  $\text{N}_2\text{O}_4$  stream was removed from the stirring reaction mixture and the mixture was allowed to stir for 5 minutes at 0 °C and an additional 1 hour at room temperature. The solvent and excess gas was removed *in vacuo* affording 17.52 g of bis-1,2,4-triazoline-3,5-dione (bis-TAD) (104).  $^1\text{H}$  NMR gives spectra consistent with pure product.

30 Derivatization of an aminomethylated polystyrene resin with hexadiene. Scheme 29 illustrates the derivatization of an aminomethylated polystyrene resin with hexadiene. Three different amino methylated resins: 1% crosslinked polystyrene(1% DVB-